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(54) Title: ANTISENSE MODULATION OF APOLIPOPROTEIN B EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of apolipoprotein B. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding apolipoprotein B. Methods of using these compounds for modulation of apolipoprotein B expression and for treatment of diseases associated with expression of apolipoprotein B are provided.

ANTISENSE MODULATION OF APOLIPOPROTEIN B EXPRESSION

This application is a continuation-in-part of PCT application US03/15493,filed on May 15, 2003, which claims priority to U.S. provisional Application Serial No: 60/426,234, filed November 13, 2002, and which is a continuation-in-part of U.S. Application Serial No. 10/147,196 filed May 15, 2002 (Attorney Docket No. ISPH-0664) which is a continuation-in-part of U.S. Application Serial No. 10/135,985 filed April 30, 2002 (Attorney Docket No. ISPH-0663) which is a continuation-in-part of U.S. Application Serial No.09/920,033 filed August 1, 2001 (Attorney Docket No ISPH-0592).

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of apolipoprotein B. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding apolipoprotein B. Such compounds have been shown to modulate the expression of apolipoprotein B.

BACKGROUND OF THE INVENTION

Lipoproteins are globular, micelle-like particles that consist of a non-polar core of acylglycerols and cholesteryl esters surrounded by an amphiphilic coating of protein, phospholipid and cholesterol. Lipoproteins have been classified into five broad categories on the basis of their functional and physical properties: chylomicrons, which transport dietary lipids from intestine to tissues; very low density lipoproteins (VLDL); intermediate density

lipoproteins (IDL); low density lipoproteins (LDL); all of which transport triacylglycerols and cholesterol from the liver to tissues; and high density lipoproteins (HDL), which transport endogenous cholesterol from tissues to the liver.

Lipoprotein particles undergo continuous metabolic processing and have variable properties and compositions. Lipoprotein densities increase without decreasing particle diameter because the density of their outer coatings is less than that of the inner core. The protein components of lipoproteins are known as apoliproteins. At least nine apolipoproteins are distributed in significant amounts among the various human lipoproteins.

Apolipoprotein B (also known as ApoB, apolipoprotein B-100; ApoB-100, apolipoprotein B-48; ApoB-48 and Ag(x) antigen), is a large glycoprotein that serves an indispensable role in the assembly and secretion of lipids and in the transport and receptor-mediated uptake and delivery of distinct classes of lipoproteins. The importance of apolipoprotein B spans a variety of functions, from the absorption and processing of dietary lipids to the regulation of circulating lipoprotein levels (Davidson and Shelness, *Annu. Rev. Nutr.*, **2000**, *20*, 169-193). This latter property underlies its relevance in terms of atherosclerosis susceptibility, which is highly correlated with the ambient concentration of apolipoprotein B-containing lipoproteins (Davidson and Shelness, *Annu. Rev. Nutr.*, **2000**, *20*, 169-193).

Two forms of apolipoprotein B exist in mammals. ApoB-100 represents the full-length protein containing 4536 amino acid residues synthesized exclusively in the human liver (Davidson and Shelness, *Annu. Rev. Nutr.*, **2000**, *20*,

169-193). A truncated form known as ApoB-48 is colinear with the amino terminal 2152 residues and is synthesized in the small intestine of all mammals (Davidson and Shelness, *Annu. Rev. Nutr.*, **2000**, *20*, 169-193).

ApoB-100 is the major protein component of LDL and contains the domain required for interaction of this lipoprotein species with the LDL receptor. In addition, ApoB-100 contains an unpaired cysteine residue which mediates an interaction with apolipoprotein(a) and generates another distinct atherogenic lipoprotein called Lp(a) (Davidson and Shelness, *Annu. Rev. Nutr.*, **2000**, *20*, 169-193).

In humans, ApoB-48 circulates in association with chylomicrons and chylomicron remnants and these particles are cleared by a distinct receptor known as the LDL-receptor-related protein (Davidson and Shelness, *Annu. Rev. Nutr.*, **2000**, *20*, 169-193). ApoB-48 can be viewed as a crucial adaptation by which dietary lipid is delivered from the small intestine to the liver, while ApoB-100 participates in the transport and delivery of endogenous plasma cholesterol (Davidson and Shelness, *Annu. Rev. Nutr.*, **2000**, *20*, 169-193).

The basis by which the common structural gene for apolipoprotein B produces two distinct protein isoforms is a process known as RNA editing. A site specific cytosine-to-uracil editing reaction produces a UAA stop codon and translational termination of apolipoprotein B to produce ApoB-48 (Davidson and Shelness, *Annu. Rev. Nutr.*, **2000**, *20*, 169-193).

Apolipoprotein B was cloned in 1985 (Law et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1985**, *82*, 8340-8344) and mapped

to chromosome 2p23-2p24 in 1986 (Deeb et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1986**, *83*, 419-422).

Disclosed and claimed in US patent 5,786,206 are methods and compositions for determining the level of low density lipoproteins (LDL) in plasma which include isolated DNA sequences encoding epitope regions of apolipoprotein B-100 (Smith et al., **1998**).

Transgenic mice expressing human apolipoprotein B and fed a high-fat diet were found to develop high plasma cholesterol levels and displayed an 11-fold increase in atherosclerotic lesions over non-transgenic littermates (Kim and Young, *J. Lipid Res.*, **1998**, *39*, 703-723; Nishina et al., *J. Lipid Res.*, **1990**, *31*, 859-869).

In addition, transgenic mice expressing truncated forms of human apolipoprotein B have been employed to identify the carboxyl-terminal structural features of ApoB-100 that are required for interactions with apolipoprotein(a) to generate the Lp(a) lipoprotein particle and to investigate structural features of the LDL receptor-binding region of ApoB-100 (Kim and Young, *J. Lipid Res.*, **1998**, *39*, 703-723; McCormick et al., *J. Biol. Chem.*, **1997**, *272*, 23616-23622).

Apolipoprotein B knockout mice (bearing disruptions of both ApoB-100 and ApoB-48) have been generated which are protected from developing hypercholesterolemia when fed a high-fat diet (Farese et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1995**, *92*, 1774-1778; Kim and Young, *J. Lipid Res.*, **1998**, *39*, 703-723). The incidence of atherosclerosis has been investigated in mice expressing exclusively ApoB-100 or ApoB-48 and susceptibility to atherosclerosis was found to be dependent on total cholesterol levels. Whether the mice synthesized ApoB-100 or ApoB-48 did not affect the

extent of the atherosclerosis, indicating that there is probably no major difference in the intrinsic atherogenicity of ApoB-100 versus ApoB-48 (Kim and Young, *J. Lipid Res.*, **1998**, *39*, 703-723; Veniant et al., *J. Clin. Invest.*, **1997**, *100*, 180-188).

Elevated plasma levels of the ApoB-100-containing lipoprotein Lp(a) are associated with increased risk for atherosclerosis and its manifestations, which may include hypercholesterolemia (Seed et al., *N. Engl. J. Med.*, **1990**, *322*, 1494-1499), myocardial infarction (Sandkamp et al., *Clin. Chem.*, **1990**, *36*, 20-23), and thrombosis (Nowak-Gottl et al., *Pediatrics*, **1997**, *99*, E11).

The plasma concentration of Lp(a) is strongly influenced by heritable factors and is refractory to most drug and dietary manipulation (Katan and Beynen, *Am. J. Epidemiol.*, **1987**, *125*, 387-399; Vessby et al., *Atherosclerosis*, **1982**, *44*, 61-71). Pharmacologic therapy of elevated Lp(a) levels has been only modestly successful and apheresis remains the most effective therapeutic modality (Hajjar and Nachman, *Annu. Rev. Med.*, **1996**, *47*, 423-442).

Disclosed and claimed in US patent 6,156,315 and the corresponding PCT publication WO 99/18986 is a method for inhibiting the binding of LDL to blood vessel matrix in a subject, comprising administering to the subject an effective amount of an antibody or a fragment thereof, which is capable of binding to the amino-terminal region of apolipoprotein B, thereby inhibiting the binding of low density lipoprotein to blood vessel matrix (Goldberg and Pillarisetti, **2000**; Goldberg and Pillarisetti, **1999**).

Disclosed and claimed in US patent 6,096,516 are vectors containing cDNA encoding murine recombinant antibodies which bind to human ApoB-100 for the purpose of

for diagnosis and treatment of cardiovascular diseases (Kwak et al., **2000**).

Disclosed and claimed in European patent application EP 911344 published April 28, 1999 (and corresponding to U.S. Patent 6,309,844) is a monoclonal antibody which specifically binds to ApoB-48 and does not specifically bind to ApoB-100, which is useful for diagnosis and therapy of hyperlipidemia and arterial sclerosis (Uchida and Kurano, **1998**).

Disclosed and claimed in PCT publication WO 01/30354 are methods of treating a patient with a cardiovascular disorder, comprising administering a therapeutically effective amount of a compound to said patient, wherein said compound acts for a period of time to lower plasma concentrations of apolipoprotein B or apolipoprotein B-containing lipoproteins by stimulating a pathway for apolipoprotein B degradation (Fisher and Williams, **2001**).

Disclosed and claimed in US patent 5,220,006 is a cloned *cis*-acting DNA sequence that mediates the suppression of atherogenic apolipoprotein B (Ross et al., **1993**).

Disclosed and claimed in PCT publication WO 01/12789 is a ribozyme which cleaves ApoB-100 mRNA specifically at position 6679 (Chan et al., **2001**).

To date, strategies aimed at inhibiting apolipoprotein B function have been limited to Lp(a) apheresis, antibodies, antibody fragments and ribozymes. However, with the exception of Lp(a) apheresis, these investigative strategies are untested as therapeutic protocols. Consequently, there remains a long felt need for additional agents capable of effectively inhibiting apolipoprotein B function.

Antisense technology is emerging as an effective means of reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic and research applications involving modulation of apolipoprotein B expression.

The present invention provides compositions and methods for modulating apolipoprotein B expression, including inhibition of the alternative isoform of apolipoprotein B, ApoB-48.

SUMMARY OF THE INVENTION

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding apolipoprotein B, and which modulate the expression of apolipoprotein B. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of apolipoprotein B in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of apolipoprotein B by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

In particular, the invention provides a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with and inhibits the expression of a nucleic acid molecule encoding apolipoprotein B, said

compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOs: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and 845-854.

The invention further provides compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding apolipoprotein B, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOs: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and 845-854, said active site being a region in said nucleic acid wherein binding of said compound to said site significantly inhibits apolipoprotein B expression as compared to a control.

The invention also provides a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with said nucleic acid and inhibits expression of apolipoprotein B, wherein the apolipoprotein B is encoded by a polynucleotide selected from the group consisting of: (a) SEQ ID NO: 3 and (b) a naturally occurring variant apolipoprotein B-encoding polynucleotide that hybridizes to the complement of the polynucleotide of (a) under stringent conditions, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOs: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and 845-854.

In another aspect the invention provides a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with said nucleic acid and inhibits expression of apolipoprotein B, wherein the apolipoprotein B is encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 17, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOs: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and 845-854.

The invention also provides a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with an active site in said nucleic acid and inhibits expression of apolipoprotein B, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOs: 127-134, 136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and 845-854, said active site being a region in said nucleic acid wherein binding of said compound to said site significantly inhibits apolipoprotein B expression as compared to a control.

In another aspect the invention provides an oligonucleotide mimetic compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with said nucleic acid and inhibits expression of apolipoprotein B, said compound comprising at least 8 contiguous nucleobases of any one of SEQ ID NOs: 127-134,

136, 138-174, 176-317, 319-321, 323-333, 335-339, 341-374, 376-416, 418-500, 502-510, 512-804, 815, 816, 819-821, 824, 825, 827, 828, 830, 831, 833-835, 837-839, 842, 843, and 845-854.

In another aspect, the invention provides an antisense compound 8 to 50 nucleobases in length, wherein said compound specifically hybridizes with nucleotides 2920-3420 as set forth in SEQ ID NO:3 and inhibits expression of mRNA encoding human apolipoprotein B after 16 to 24 hours by at least 30% in 80% confluent HepG2 cells in culture at a concentration of 150 nM. In preferred embodiments, the antisense compound 8 to 50 nucleobases in length specifically hybridizes with nucleotides 3230-3288 as set forth in SEQ ID NO:3 and inhibits expression of mRNA encoding human apolipoprotein B after 16 to 24 hours by at least 30% in 80% confluent HepG2 cells in culture at a concentration of 150 nM. In another aspect, the compounds inhibits expression of mRNA encoding apolipoprotein B by at least 50%, after 16 to 24 hours in 80% confluent HepG2 cells in culture at a concentration of 150 nM.

In one aspect, the compounds of the invention are targeted to a nucleic acid molecule encoding apolipoprotein B, wherein said compound specifically hybridizes with and inhibits expression of the long form of apolipoprotein B, ApoB-100. In another aspect, the compounds specifically hybridizes with said nucleic acid and inhibits expression of mRNA encoding apolipoprotein B by at least 5% in 80% confluent HepG2 cells in culture at an optimum concentration. In yet another aspect, the compounds inhibits expression of mRNA encoding apolipoprotein B by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, or at least 50%.

In one aspect, the compounds are antisense oligonucleotides, and in one embodiment the compound has a sequence comprising SEQ ID NO: 224, the antisense oligonucleotide hybridizes with a region complementary to SEQ ID NO: 224, the compound comprises SEQ ID NO: 224, the compound consists essentially of SEQ ID NO: 224 or the compound consists of SEQ ID NO: 224.

In another aspect, the compound has a sequence comprising SEQ ID NO: 247, the antisense oligonucleotide hybridizes with a region complementary to SEQ ID NO: 247, the compound comprises SEQ ID NO: 247, the compound consists essentially of SEQ ID NO: 247 or the compound consists of SEQ ID NO: 247.

In another aspect, the compound has a sequence comprising SEQ ID NO: 319, the antisense oligonucleotide hybridizes with a region complementary to SEQ ID NO: 319, the compound comprises SEQ ID NO: 319, the compound consists essentially of SEQ ID NO: 319 or the compound consists of SEQ ID NO: 319.

In one embodiment, the compounds comprise at least one modified internucleoside linkage, and in another embodiment, the modified internucleoside linkage is a phosphorothioate linkage.

In another aspect, the compounds comprise at least one modified sugar moiety, and in one aspect, the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

In another embodiment, the compounds comprise at least one modified nucleobase, and in one aspect, the modified nucleobase is a 5-methylcytosine.

In yet another aspect, the compounds are chimeric oligonucleotides. Preferred chimeric compounds include those having one or more phosphorothioate linkages and

further comprising 2'-methoxyethoxyl nucleotide wings and a ten nucleobase 2'-deoxynucleotide gap.

In another aspect, the compounds specifically hybridizes with and inhibits the expression of a nucleic acid molecule encoding an alternatively spliced form of apolipoprotein B.

The invention also provide compositions comprising a compound of the invention and a pharmaceutically acceptable carrier or diluent. In one aspect, the composition further comprises a colloidal dispersion system, and in another aspect, the compound in the composition is an antisense oligonucleotide. In certain embodiments, the composition comprises an antisense compound of the invention hybridized to a complementary strand. Hybridization of the antisense strand can form one or more blunt ends or one or more overhanging ends. In some embodiments, the overhanging end comprises a modified base.

The invention further provides methods of inhibiting the expression of apolipoprotein B in cells or tissues comprising contacting said cells or tissues with a compound of the invention so that expression of apolipoprotein B is inhibited. Methods are also provided for treating an animal having a disease or condition associated with apolipoprotein B comprising administering to said animal a therapeutically or prophylactically effective amount of a compound of the invention so that expression of apolipoprotein B is inhibited. In various aspects, the condition is associated with abnormal lipid metabolism, the condition is associated with abnormal cholesterol metabolism, the condition is atherosclerosis, the condition is an abnormal metabolic condition, the abnormal metabolic condition is hyperlipidemia, the disease is diabetes, the

diabetes is Type 2 diabetes, the condition is obesity, and/or the disease is cardiovascular disease.

The invention also provide methods of modulating glucose levels in an animal comprising administering to said animal a compound of the invention, and in one aspect, the animal is a human. In various embodiments, the glucose levels are plasma glucose levels, the glucose levels are serum glucose levels, and/or the animal is a diabetic animal.

The invention also provides methods of preventing or delaying the onset of a disease or condition associated with apolipoprotein B in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human. In other aspects, the condition is an abnormal metabolic condition, the abnormal metabolic condition is hyperlipidemia, the disease is diabetes, the diabetes is Type 2 diabetes, the condition is obesity, the condition is atherosclerosis, the condition involves abnormal lipid metabolism, and/or the condition involves abnormal cholesterol metabolism.

The invention also provides methods of preventing or delaying the onset of an increase in glucose levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human. In other aspects, the glucose levels are serum glucose levels, and/or the glucose levels are plasma glucose levels.

The invention also provides methods of modulating serum cholesterol levels in an animal comprising administering to said animal a therapeutically or

prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human.

The invention also provides methods of modulating lipoprotein levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human. In other aspects, the lipoprotein is VLDL, the lipoprotein is HDL, and/or the lipoprotein is LDL.

The invention also provides methods of modulating serum triglyceride levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of a compound of the invention. In one aspect, the animal is a human.

The invention also proves use of a compound of the invention for the manufacture of a medicament for the treatment of a disease or condition associated with apolipoprotein B expression, a medicament for the treatment of a condition associated with abnormal lipid metabolism, a medicament for the treatment of a condition associated with abnormal cholesterol metabolism, a medicament for the treatment of atherosclerosis, a medicament for the treatment of hyperlipidemia, a medicament for the treatment of diabetes, a medicament for the treatment of Type 2 diabetes, a medicament for the treatment of obesity, a medicament for the treatment of cardiovascular disease, a medicament for preventing or delaying the onset of increased glucose levels, a medicament for preventing or delaying the onset of increased serum glucose levels, a medicament for preventing or delaying the onset of increased plasma glucose levels, a medicament for the modulation of serum cholesterol levels, a medicament for

the modulation of serum lipoprotein levels, a medicament for the modulation of serum VLDL levels, a medicament for the modulation of serum HDL levels, and/or a medicament for the modulation of serum LDL levels, a medicament for the modulation of serum triglyceride levels.

In another aspect, the invention provides methods of decreasing circulating lipoprotein levels comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. In another aspect, the invention provides methods of reducing lipoprotein transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. The invention also provides methods of reducing lipoprotein absorption/adsorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In another aspect, the invention contemplates methods of decreasing circulating triglyceride levels comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. Also provided are methods of reducing triglyceride transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. The invention further provides methods of reducing triglyceride absorption/adsorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In another aspect, the invention provides methods of decreasing circulating cholesterol levels, including cholesteryl esters and/or unesterified cholesterol, comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. Also contemplated are methods of reducing cholesterol transport, including cholesteryl esters and/or unesterified cholesterol, comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. The invention also provides methods of reducing cholesterol absorption/adsorption, including cholesteryl esters and/or unesterified cholesterol, comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In another aspect, the invention provides methods of decreasing circulating lipid levels comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. The invention also provides methods of reducing lipid transport in plasma comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. In addition, the invention provides methods of reducing lipid absorption/adsorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

The invention further contemplates methods of decreasing circulating dietary lipid levels comprising the step of administering to an individual an amount of a

compound of the invention sufficient to reduce apolipoprotein B expression. Also provided are methods of reducing dietary lipid transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, as well as methods of reducing dietary lipid absorption/adsorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In another aspect, the invention provides methods of decreasing circulating fatty acid levels comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. The invention also provides methods of reducing fatty acid transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. Also contemplated are methods of reducing fatty acid absorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

The invention also provides methods of decreasing circulating acute phase reactants comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. In another aspect, the invention provides methods of reducing acute phase reactants transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, as well as methods of reducing acute phase reactants absorption comprising the step of

administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In another aspect, the invention provides methods of decreasing circulating chylomicrons comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, methods of reducing chylomicron transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, and methods of reducing chylomicron absorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

The invention further provides methods of decreasing circulating chylomicron remnant particles comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, methods of reducing chylomicron remnant transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, and methods of reducing chylomicron remnant absorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

The invention further contemplates methods of decreasing circulating VLDL, IDL, LDL, and/or HDL comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression. Likewise, the invention provides methods of reducing VLDL, IDL, LDL, and/or HDL

transport comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression, in addition to methods of reducing VLDL, IDL, LDL, and/or HDL absorption comprising the step of administering to an individual an amount of a compound of the invention sufficient to reduce apolipoprotein B expression.

In still another aspect, the invention provides methods of treating a condition associated with apolipoprotein B expression comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression, said condition selected from hyperlipoproteinemia, familial type 3 hyperlipoprotienemia (familial dysbetalipoproteinemia), and familial hyperalphalipoprotienemia; hyperlipidemia, mixed hyperlipidemias, multiple lipoprotein-type hyperlipidemia, and familial combined hyperlipidemia; hypertriglyceridemia, familial hypertriglyceridemia, and familial lipoprotein lipase; hypercholesterolemia, familial hypercholesterolemia, polygenic hypercholesterolemia, and familial defective apolipoprotein B; cardiovascular disorders including atherosclerosis and coronary artery disease; peripheral vascular disease; von Gierke's disease (glycogen storage disease, type I); lipodystrophies (congenital and acquired forms); Cushing's syndrome; sexual ateloitic dwarfism (isolated growth hormone deficiency); diabetes mellitus; hyperthyroidism; hypertension; anorexia nervosa; Werner's syndrome; acute intermittent porphyria; primary biliary cirrhosis; extrahepatic biliary obstruction; acute hepatitis; hepatoma; systemic lupus erythematosis; monoclonal gammopathies (including myeloma,

multiple myeloma, macroglobulinemia, and lymphoma); endocrinopathies; obesity; nephrotic syndrome; metabolic syndrome; inflammation; hypothyroidism; uremia (hyperurecemia); impotence; obstructive liver disease; idiopathic hypercalcemia; dysglobulinemia; elevated insulin levels; Syndrome X; Dupuytren's contracture; and Alzheimer's disease and dementia.

The invention also provides methods of reducing the risk of a condition comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression, said condition selected from pregnancy; intermittent claudication; gout; and mercury toxicity and amalgam illness.

The invention further provides methods of inhibiting cholesterol particle binding to vascular endothelium comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression, and as a result, the invention also provides methods of reducing the risk of: (i) cholesterol particle oxidization; (ii) monocyte binding to vascular endothelium; (iii) monocyte differentiation into macrophage; (iv) macrophage ingestion of oxidized lipid particles and release of cytokines (including, but limited to IL-1,TNF-alpha, TGF-beta); (v) platelet formation of fibrous fibrofatty lesions and inflammation; (vi) endothelium lesions leading to clots; and (vii) clots leading to myocardial infarction or stroke, also comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression.

The invention also provides methods of reducing hyperlipidemia associated with alcoholism, smoking, use of oral contraceptives, use of glucocorticoids, use of beta-adrenergic blocking agents, or use of isotretinion (13-cis-retinoic acid) comprising the step of administering to an individual an amount of a compound of the invention sufficient to inhibit apolipoprotein B expression.

In certin aspects, the invention provides an antisense oligonucleotide compound 8 to 50 nucleobases in length comprising at least 8 contiguous nucleotides of SEQ ID NO:247 and having a length from at least 12 or at least 14 to 30 nucleobases.

In a further aspect, the invention provides an antisense oligonucleotide compound 20 nucleobases in length having a sequence of nucleobases as set forth in SEQ ID NO:247 and comprising 5-methylcytidine at nucleobases 2, 3, 5, 9, 12, 15, 17, 19, and 20, wherein every internucleoside linkage is a phosphothioate linkage, nucleobases 1-5 and 16-20 comprise a 2'-methoxyethoxyl modification, and nucleobases 6-15 are deoxynucleotides.

In another aspect, the invention provides a compound comprising a first nucleobase strand, 8 to 50 nucleobases in length and comprising a sequence of at least 8 contiguous nucleobases of the sequence set forth in SEQ ID NO:3, hybridized to a second nucleobase strand, 8 to 50 nucleobases in length and comprising a sequence sufficiently complementary to the first strand so as to permit stable hybridization, said compound inhibiting expression of mRNA encoding human apolipoprotein B after 16 to 24 hours by at least 30% or by at least 50% in 80% confluent HepG2 cells in culture at a concentration of 100 nM.

Further provided is a vesicle, such as a liposome, comprising a compound or composition of the invention

Preferred methods of administration of the compounds or compositions of the invention to an animal are intravenously, subcutaneously, or orally. Administrations can be repeated.

In another aspect, the invention provides a method of reducing lipoprotein(a) secretion by hepatocytes comprising (a)contacting hepatocytes with an amount of a composition comprising a non-catalytic compound 8 to 50 nucleobases in length that specifically hybridizes with mRNA encoding human apolipoprotein B and inhibits expression of the mRNA after 16 to 24 hours by at least 30% or at least 50% in 80% confluent HepG2 cells in culture at a concentration of 150 nM, wherein said amount is effective to inhibit expression of apolipoprotein B in the hepatocytes; and (b) measuring lipoprotein(a) secretion by the hepatocytes.

The invention further provides a method of a treating a condition associated with apolipoprotein B expression in a primate, such as a human, comprising administering to the primate a therapeutically or prophylactically effective amount of a non-catalytic compound 8 to 50 nucleobases in length that specifically hybridizes with mRNA encoding human apolipoprotein B and inhibits expression of the mRNA after 16 to 24 hours by at least 30% or by at least 50% in 80% confluent HepG2 cells in culture at a concentration of 150 nM.

The invention provides a method of reducing apolipoprotein B expression in the liver of an animal, comprising administering to the animal between 2 mg/kg and 20 mg/kg of a non-catalytic compound 8 to 50 nucleobases in length that specifically hybridizes with mRNA encoding

human apolipoprotein B by at least 30% or by at least 50% in 80% confluent HepG2 cells in culture at a concentration of 150 nM.

Also provided is a method of making a compound of the invention comprising specifically hybridizing *in vitro* a first nucleobase strand comprising a sequence of at least 8 contiguous nucleobases of the sequence set forth in SEQ ID NO:3 to a second nucleobase strand comprising a sequence sufficiently complementary to said first strand so as to permit stable hybridization.

The invention further provides use of a compound of the invention in the manufacture of a medicament for the treatment of any and all conditions disclosed herein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding apolipoprotein B, ultimately modulating the amount of apolipoprotein B produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding apolipoprotein B. As used herein, the terms "target nucleic acid" and "nucleic acid encoding apolipoprotein B" encompass DNA encoding apolipoprotein B, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with

include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of apolipoprotein B. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding apolipoprotein B. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame

(ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding apolipoprotein B, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly,

the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e.,

intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a

sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular

genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene expression)(Madden, et al., *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81), protein arrays and proteomics (Celis, et al.,

FEBS Lett., **2000**, 480, 2-16; Jungblut, et al., *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Larsson, et al., *J. Biotechnol.*, **2000**, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, **2000**, 286, 91-98; Larson, et al., *Cytometry*, **2000**, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, **1998**, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, **1999**, 35, 1895-904) and mass spectrometry methods (reviewed in (To, *Comb. Chem. High Throughput Screen*, **2000**, 3, 235-41).

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. Thus, this term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages (RNA and DNA) as well as oligonucleotides having

non-naturally-occurring portions which function similarly (oligonucleotide mimetics). Oligonucleotide mimetics are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12, about 14, about 20 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression. In preferred embodiments, the antisense compound is non-catalytic oligonucleotide, *i.e.*, is not dependent on a catalytic property of the oligonucleotide for its modulating activity. Antisense compounds of the invention can include double-stranded molecules wherein a first strand is stably hybridized to a second strand.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further

include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and

aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone

backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the

preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$]of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino,

polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylamino-ethoxyethoxy (also known in the art as 2'-O-dimethylamino-ethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_2)_2$, also described in examples hereinbelow.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-$CH=CH_2$), 2'-O-allyl (2'-O-CH_2-$CH=CH_2$) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5'

terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-

deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more

particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention,

include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J.*

Pharmacol. Exp. Ther., **1996**, *277*, 923-937.

Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than

one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides,

oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting

formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the

desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric

acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid,

hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of apolipoprotein B is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding apolipoprotein B, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding apolipoprotein B can be detected by means known in

the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of apolipoprotein B in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery

agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which

oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate,. Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include

poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines,

pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999) each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit

dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 µm in diameter (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-

in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., **1988**, volume 1, p. 199). Surfactants are

typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York,

N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for

their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, *in: Controlled Release of Drugs: Polymers*

and Aggregate Systems, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol

decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms,

improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385; Ho *et al.*, *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome

formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome,

the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, ***1987,*** *147*, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, **1992,** *19*, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (*e.g.* as a solution or as an emulsion) were ineffective (Weiner *et al.*, *Journal of Drug Targeting*, **1992**, *2*, 405-410). Further, an additional study

tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al.*, *Antiviral Research*, **1992,** *18*, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P.Pharma. Sci.*, **1994**, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized

lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters*, **1987**, *223*, 42; Wu *et al.*, *Cancer Research*, **1993**, *53*, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, **1987**, *507*, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, **1988**, *85*, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al.*).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn.*, **1980**, *53*, 2778) described liposomes comprising a nonionic detergent, $2C_{12}15G$, that contains a PEG moiety. Illum *et al.* (*FEBS Lett.*, **1984**, *167*, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Lett.*, **1990**, *268*, 235) described experiments demonstrating that

liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta,* **1990,** *1029*, 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et*

al. discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.* they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic

products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In

addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm. Pharmacol.*, **1988**, *40*, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol),, dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof (*e.g.*, methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., **1992**, *44*, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, **1996**, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring

components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Swinyard, Chapter 39 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, **1992**, *263*, 25; Yamashita *et al.*, *J. Pharm. Sci.*, **1990**, *79*, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339). Chelating agents of the invention include but are not

limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Buur *et al.*, *J. Control Rel.*, **1990**, *14*, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including

glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, **1995**, *5*, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6*, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable

solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as

alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Pulsatile Delivery

The compounds of the present invention may also be administered by pulsatile delivery. "Pulsatile delivery" refers to a pharmaceutical formulations that delivers a first pulse of drug combined with a penetration enhancer and a second pulse of penetration enhancer to promote absorption of drug which is not absorbed upon release with the first pulse of penetration enhancer.

One embodiment of the present invention is a delayed release oral formulation for enhanced intestinal drug absorption, comprising:

(a) a first population of carrier particles comprising said drug and a penetration enhancer, wherein said drug and said penetration enhancer are released at a first location in the intestine; and

(b) a second population of carrier particles comprising a penetration enhancer and a delayed release coating or matrix, wherein the penetration enhancer is released at a second location in the intestine downstream

from the first location, whereby absorption of the drug is enhanced when the drug reaches the second location.

Alternatively, the penetration enhancer in (a) and (b) is different.

This enhancement is obtained by encapsulating at least two populations of carrier particles. The first population of carrier particles comprises a biologically active substance and a penetration enhancer, and the second (and optionally additional) population of carrier particles comprises a penetration enhancer and a delayed release coating or matrix.

A "first pass effect" that applies to orally administered drugs is degradation due to the action of gastric acid and various digestive enzymes. One means of ameliorating first pass clearance effects is to increase the dose of administered drug, thereby compensating for proportion of drug lost to first pass clearance. Although this may be readily achieved with i.v. administration by, for example, simply providing more of the drug to an animal, other factors influence the bioavailability of drugs administered via non-parenteral means. For example, a drug may be enzymatically or chemically degraded in the alimentary canal or blood stream and/or may be impermeable or semipermeable to various mucosal membranes.

It is also contemplated that these pharmaceutical compositons are capable of enhancing absorption of biologically active substances when administered via the rectal, vaginal, nasal or pulmonary routes. It is also contemplated that release of the biologically active substance can be achieved in any part of the gastrointestinal tract.

Liquid pharmaceutical compositions of oligonucleotide can be prepared by combining the oligonucleotide with a suitable vehicle, for example sterile pyrogen free water, or saline solution. Other therapeutic compounds may optionally be included.

The present invention also contemplates the use of solid particulate compositions. Such compositions preferably comprise particles of oligonucleotide that are of respirable size. Such particles can be prepared by, for example, grinding dry oligonucleotide by conventional means, fore example with a mortar and pestle, and then passing the resulting powder composition through a 400 mesh screen to segregate large particles and agglomerates. A solid particulate composition comprised of an active oligonucleotide can optionally contain a dispersant which serves to facilitate the formation of an aerosol, for example lactose.

In accordance with the present invention, oligonucleotide compositions can be aerosolized. Aerosolization of liquid particles can be produced by any suitable means, such as with a nebulizer. See, for example, U.S. Patent No. 4,501,729. Nebulizers are commercially available devices which transform solutions or suspensions into a therapeutic aerosol mist either by means of acceleration of a compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable nebulizers include those sold by Blairex® under the name PARI LC PLUS, PARI DURA-NEB 2000, PARI-BABY Size, PARI PRONEB Compressor with LC PLUS, PARI WALKHALER Compressor/Nebulizer System, PARI LC PLUS Reusable Nebulizer, and PARI LC Jet+ ®Nebulizer.

Exemplary formulations for use in nebulizers consist of an oligonucleotide in a liquid, such as sterile, pyragen free water, or saline solution, wherein the oligonucleotide comprises up to about 40% w/w of the formulation. Preferably, the oligonucleotide comprises less than 20% w/w. If desired, further additives such as preservatives (for example, methyl hydroxybenzoate) antioxidants, and flavoring agents can be added to the composition.

Solid particles comprising an oligonucleotide can also be aerosolized using any solid particulate medicament aerosol generator known in the art. Such aerosol generators produce respirable particles, as described above, and further produce reproducible metered dose per unit volume of aerosol. Suitable solid particulate aerosol generators include insufflators and metered dose inhalers. Metered dose inhalers are used in the art and are useful in the present invention.

Preferably, liquid or solid aerosols are produced at a rate of from about 10 to 150 liters per minute, more preferably from about 30 to 150 liters per minute, and most preferably about 60 liters per minute.

Enhanced bioavailability of biologically active substances is also achieved via the oral administration of the compositions and methods of the present invention. The term "bioavailability" refers to a measurement of what portion of an administered drug reaches the circulatory system when a non-parenteral mode of administration is used to introduce the drug into an animal.

Penetration enhancers include, but are not limited to, members of molecular classes such as surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactant molecules. (Lee *et al.*, *Critical Reviews in*

Therapeutic Drug Carrier Systems, 1991, p. 92). Carriers are inert molecules that may be included in the compositions of the present invention to interfere with processes that lead to reduction in the levels of bioavailable drug.

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp. 1206-1228, Berkow *et al.*, eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and

corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly,

monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

Combination Therapy

The invention also provides methods of combination therapy, wherein one or more compounds of the invention and one or more other therapeutic/prophylactic compounds are administered treat a condition and/or disease state as described herein. In various aspects, the compound(s) of the invention and the therapeutic/prophylactic compound(s) are co-administered as a mixture or administered individually. In one aspect, the route of administration is the same for the compound(s) of the invention and the therapeutic/prophylactic compound(s), while in other aspects, the compound(s) of the invention and the therapeutic/prophylactic compound(s) are administered by a different routes. In one embodiment, the dosages of the compound(s) of the invention and the therapeutic/prophylactic compound(s) are amounts that are therapeutically or prophylactically effective for each compound when administered individually. Alternatively, the combined administration permits use of lower dosages than would be required to achieve a therapeutic or prophylactic effect if administered individually, and such methods are

useful in decreasing one or more side effects of the reduced-dose compound.

In one aspect, a compound of the present invention and one or more other therapeutic/prophylactic compound(s) effective at treating a condition are administered wherein both compounds act through the same or different mechanisms. Therapeutic/prophylactic compound(s) include, but are not limited to, bile salt sequestering resins (*e.g.*, cholestyramine, colestipol, and colesevelam hydrochloride), HMGCoA-redectase inhibitors (*e.g.*, lovastatin, cerivastatin, prevastatin, atorvastatin, simvastatin, and fluvastatin), nicotinic acid, fibric acid derivatives (*e.g.*, clofibrate, gemfibrozil, fenofibrate, bezafibrate, and ciprofibrate), probucol, neomycin, dextrothyroxine, plant-stanol esters, cholesterol absorption inhibitors (*e.g.*, ezetimibe), implitapide, inhibitors of bile acid transporters (apical sodium-dependent bile acid transporters), regulators of hepatic CYP7a, estrogen replacement therapeutics (*e.g.*, tamoxigen), and anti-inflammatories (*e.g.*, glucocorticoids).

Accordingly, the invention further provides use of a compound of the invention and one or more other therapeutic/prophylactic compound(s) as described herein in the manufacture of a medicament for the treatment and/or prevention of a disease or condition as described herein.

Targeted Delivery

In another aspect, methods are provided to target a compound of the invention to a specific tissue, organ or location in the body. Exemplary targets include liver, lung, kidney, heart, and atherosclerotic plaques within a

blood vessel. Methods of targeting compounds are well known in the art.

In one embodiment, the compound is targeted by direct or local administration. For example, when targeting a blood vessel, the compound is administered directly to the relevant portion of the vessel from inside the lumen of the vessel, *e.g.*, single balloon or double balloon catheter, or through the adventitia with material aiding slow release of the compound, *e.g.*, a pluronic gel system as described by Simons *et al.*, *Nature 359*: 67-70 (**1992**). Other slow release techniques for local delivery of the compound to a vessel include coating a stent with the compound. Methods of delivery of antisense compounds to a blood vessel are disclosed in U.S. Patent No. 6,159,946, which is incorporated by reference in its entirety.

When targeting a particular tissue or organ, the compound may be administered in or around that tissue or organ. For example, U.S. Patent No. 6,547,787, incorporated herein by reference in its entirety, discloses methods and devices for targeting therapeutic agents to the heart. In one aspect, administration occurs by direct injection or by injection into a blood vessel associated with the tissue or organ. For example, when targeting the liver, the compound may be administered by injection or infusion through the portal vein.

In another aspect, methods of targeting a compound are provided which include associating the compound with an agent that directs uptake of the compound by one or more cell types. Exemplary agents include lipids and lipid-based structures such as liposomes generally in combination with an organ- or tissue-specific targeting moiety such as, for example, an antibody, a cell surface receptor, a ligand

for a cell surface receptor, a polysaccharide, a drug, a hormone, a hapten, a special lipid and a nucleic acid as described in U. S. Patent No. 6,495,532, the disclosure of which is incorporated herein by reference in its entirety. U.S. Pat. No. 5,399,331, the disclosure of which is incorporated herein by reference in its entirety, describes the coupling of proteins to liposomes through use of a crosslinking agent having at least one maleimido group and an amine reactive function; U.S. Pat. Nos. 4,885,172, 5,059,421 and 5,171,578, the disclosures of which are incorporated herein by reference in their entirety, describe linking proteins to liposomes through use of the glycoprotein streptavidin and coating targeting liposomes with polysaccharides. Other lipid based targeting agents include, for example, micelle and crystalline products as described in U.S. Patent No. 6,217,886, the disclosure of which is incorporated herein by reference in its entirety.

In another aspect, targeting agents include porous polymeric microspheres which are derived from copolymeric and homopolymeric polyesters containing hydrolyzable ester linkages which are biodegradable, as described in U.S. No. Patent 4,818,542, the disclosure of which is incorporated herein by reference in its entirety. Typical polyesters include polyglycolic (PGA) and polylactic (PLA) acids, and copolymers of glycolide and L(-lactide) (PGL), which are particularly suited for the methods and compositions of the present invention in that they exhibit low human toxicity and are biodegradable. The particular polyester or other polymer, oligomer, or copolymer utilized as the microspheric polymer matrix is not critical and a variety of polymers may be utilized depending on desired porosity, consistency, shape and size distribution. Other

biodegradable or bioerodable polymers or copolymers include, for example, gelatin, agar, starch, arabinogalactan, albumin, collagen, natural and synthetic materials or polymers, such as, poly(ε-caprolactone), poly(ε-caprolactone-CO-lactic acid), poly(ε-caprolactone-CO-glycolic acid), poly(β-hydroxy butyric acid), polyethylene oxide, polyethylene, poly(alkyl-2-cyanoacrylate), (*e.g.*, methyl, ethyl, butyl), hydrogels such as poly(hydroxyethyl methacrylate), polyamides (*e.g.*, polyacrylamide), poly(amino acids) (*i.e.*, L-leucine, L-aspartic acid, β-methyl-L-aspartate, β-benzyl-L-aspartate, glutamic acid), poly(2-hydroxyethyl DL-aspartamide), poly(ester urea), poly(L-phenylalanine/ethylene glycol/1,6-diisocyanatohexane) and poly(methyl methacrylate). The exemplary natural and synthetic polymers suitable for targeted delivery are either readily available commercially or are obtainable by condensation polymerization reactions from the suitable monomers or, comonomers or oligomers.

In still another embodiment, U.S. Patent No. 6,562,864, the disclosure of which is incorporated herein by reference in its entirety, describes catechins, including epi and other carbo-cationic isomers and derivatives thereof, which as monomers, dimers and higher multimers can form complexes with nucleophilic and cationic bioactive agents for use as delivery agents. Catechin multimers have a strong affinity for polar proteins, such as those residing in the vascular endothelium, and on cell/organelle membranes and are particularly useful for targeted delivery of bioactive agents to select sites *in vivo*. In treatment of vascular diseases and disorders, such as atherosclerosis and coronary artery disease, delivery agents include substituted catechin multimers, including

amidated catechin multimers which are formed from reaction between catechin and nitrogen containing moities such as ammonia.

Other targeting strategies of the invention include ADEPT (antibody-directed enzyme prodrug therapy), GDEPT (gene-directed EPT) and VDEPT (virus-directed EPT) as described in U.S. Patent No. 6,433,012, the disclosure of which is incorporated herein by reference in its entirety.

The present invention further provides medical devices and kits for targeted delivery, wherein the device is, for example, a syringe, stent, or catheter. Kits include a device for administering a compound and a container comprising a compound of the invention. In one aspect, the compound is preloaded into the device. In other embodiments, the kit provides instructions for methods of administering the compound and dosages. U.S. patents describing medical devices and kits for delivering antisense compounds include US Patent Nos. 6,368,356; 6,344,035; 6,344,028; 6,287,285; 6,200,304; 5,824,049; 5,749,915; 5,674,242; 5,670,161; 5,609,629; 5,593,974; and 5,470,307 (all incorporated herein by reference in their entirety).

While the present invention has been described with specificity in accordance with certain embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, *36*, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro

atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups.

Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a

gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with

CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue

was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-

extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2′-O-(Aminooxyethyl) nucleoside amidites and 2′-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium

bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10° C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The

product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-*O*-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na_2SO_4, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in

MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine

(20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-([2-phthalmidoxy]ethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH_2-O-CH_2-N(CH_2)$_2$, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetra-hydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O^2-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylamino-ethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The combined CH_2Cl_2 layers are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH_2Cl_2:Et_3N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA

synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to

in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, *4*, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s

repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate

the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for

individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 7 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

T-24 cells:

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life

Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

HepG2 cells:

The human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were routinely cultured in Eagle's MEM supplemented with 10% fetal calf serum, non-essential amino acids, and 1 mM sodium pyruvate (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

AML12 cells:

The AML12 (alpha mouse liver 12) cell line was established from hepatocytes from a mouse (CD1 strain, line MT42) transgenic for human TGF alpha. Cells are cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone, and 90%; 10% fetal bovine serum. For subculturing, spent

medium is removed and fresh media of 0.25% trypsin, 0.03% EDTA solution is added. Fresh trypsin solution (1 to 2 ml) is added and the culture is left to sit at room temperature until the cells detach.

Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Primary mouse hepatocytes:

Primary mouse hepatocytes were prepared from CD-1 mice purchased from Charles River Labs (Wilmington, MA) and were routinely cultured in Hepatoyte Attachment Media (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco/Life Technologies, Gaithersburg, MD), 250nM dexamethasone (Sigma), and 10nM bovine insulin (Sigma). Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 10000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells are plated onto 100 mm or other standard tissue culture plates coated with rat tail collagen (200ug/mL) (Becton Dickinson) and treated similarly using appropriate volumes of medium and oligonucleotide.

Hep3B cells:

The human hepatocellular carcinoma cell line Hep3B was obtained from the American Type Culture Collection (Manassas, VA). Hep3B cells were routinely cultured in

Dulbeccos's MEM high glucose supplemented with 10% fetal calf serum, L-glutamine and pyridoxine hydrochloride (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 24-well plates (Falcon-Primaria #3846) at a density of 50,000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Rabbit primary hepatocytes:

Primary rabbit hepatocytes were purchased from Invitro Technologies (Gaithersburg, MD) and maintained in Dulbecco's modified Eagle's medium (Gibco). When purchased, the cells had been seeded into 96-well plates for use in RT-PCR analysis and were confluent.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly using appropriate volumes of medium and oligonucleotide.

HeLa cells:

The human epitheloid carcinoma cell line HeLa was obtained from the American Tissue Type Culture Collection (Manassas, VA). HeLa cells were routinely cultured in DMEM, high glucose (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA). Cells were seeded into 24-well plates (Falcon-Primaria #3846) at a density of 50,000 cells/well for use in RT-PCR analysis. Cells were

routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells 96-well plates (Falcon-Primaria #3872) at a density of 5,000 cells/well for use in RT-PCR analysis. For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Human mammary epithelial cells:

Normal human mammary epithelial cells (HMECs) were obtained from the American Type Culture Collection (Manassas VA). HMECs were routinely cultured in DMEM low glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #353872, BD Biosciences, Bedford, MA) at a density of 7000 cells/well for use in RT-PCR analysis. For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 µL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh

medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCC**GTCATCGCT**CCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments. The concentrations of antisense oligonucleotides used herein are from 5 nM to 300 nM.

Example 10

Analysis of oligonucleotide inhibition of apolipoprotein B

expression

Antisense modulation of apolipoprotein B expression can be assayed in a variety of ways known in the art. For example, apolipoprotein B mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, F.*M. et al., Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of apolipoprotein B can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to apolipoprotein B can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997**.

Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., **1997**.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., **1998**. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., **1991**.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, **1996**, *42*, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 µL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc.,

Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 µL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 µL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 100 µL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 µL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of

15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13

Real-time Quantitative PCR Analysis of apolipoprotein B mRNA Levels

Quantitation of apolipoprotein B mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR

primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each

dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM $MgCl_2$, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or

separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, *Analytical Biochemistry*, **1998**, *265*, 368-374.

In this assay, 175 µL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human apolipoprotein B were designed to hybridize to a human apolipoprotein B sequence, using published sequence information (GenBank accession number NM_000384.1, incorporated herein as SEQ ID NO: 3). For human apolipoprotein B the PCR primers were:
forward primer: TGCTAAAGGCACATATGGCCT (SEQ ID NO: 4)
reverse primer: CTCAGGTTGGACTCTCCATTGAG (SEQ ID NO: 5) and the PCR probe was: FAM-CTTGTCAGAGGGATCCTAACACTGGCCG-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were:
forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 8) and the PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Probes and primers to mouse apolipoprotein B were designed to hybridize to a mouse apolipoprotein B sequence, using published sequence information (GenBank accession

number M35186, incorporated herein as SEQ ID NO: 10). For mouse apolipoprotein B the PCR primers were:
forward primer: CGTGGGCTCCAGCATTCTA (SEQ ID NO: 11)
reverse primer: AGTCATTTCTGCCTTTGCGTC (SEQ ID NO: 12) and the PCR probe was: FAM-CCAATGGTCGGGCACTGCTCAA-TAMRA SEQ ID NO: 13) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For mouse GAPDH the PCR primers were:
forward primer: GGCAAATTCAACGGCACAGT (SEQ ID NO: 14)
reverse primer: GGGTCTCGCTCCTGGAAGAT (SEQ ID NO:15) and the PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC-TAMRA 3' (SEQ ID NO: 16) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of apolipoprotein B mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc,

La Jolla, CA) and then robed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human apolipoprotein B, a human apolipoprotein B specific probe was prepared by PCR using the forward primer TGCTAAAGGCACATATGGCCT (SEQ ID NO: 4) and the reverse primer CTCAGGTTGGACTCTCCATTGAG (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

To detect mouse apolipoprotein B, a human apolipoprotein B specific probe was prepared by PCR using the forward primer CGTGGGCTCCAGCATTCTA (SEQ ID NO: 11) and the reverse primer AGTCATTTCTGCCTTTGCGTC (SEQ ID NO: 12). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

Antisense inhibition of human apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides was designed to target different regions of the human apolipoprotein B RNA, using published sequence (GenBank accession number NM_000384.1, incorporated herein

as SEQ ID NO: 3). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments in which HepG2 cells were treated with 150 nM of the compounds in Table 1. If present, "N.D." indicates "no data".

Table 1

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
147780	5'UTR	3	1	CCGCAGGTCCCGGTGGGAAT	40	17
147781	5'UTR	3	21	ACCGAGAAGGGCACTCAGCC	35	18
147782	5'UTR	3	71	GCCTCGGCCTCGCGGCCCTG	67	19
147783	Start Codon	3	114	TCCATCGCCAGCTGCGGTGG	N.D.	20
147784	Coding	3	151	CAGCGCCAGCAGCGCCAGCA	70	21
147785	Coding	3	181	GCCCGCCAGCAGCAGCAGCA	29	22
147786	Coding	3	321	CTTGAATCAGCAGTCCCAGG	34	23
147787	Coding	3	451	CTTCAGCAAGGCTTTGCCCT	N.D.	24
147788	Coding	3	716	TTTCTGTTGCCACATTGCCC	95	25
147789	Coding	3	911	GGAAGAGGTGTTGCTCCTTG	24	26
147790	Coding	3	951	TGTGCTACCATCCCATACTT	33	27
147791	Coding	3	1041	TCAAATGCGAGGCCCATCTT	N.D.	28
147792	Coding	3	1231	GGACACCTCAATCAGCTGTG	26	29

147793	Coding	3	1361	TCAGGGCCACCAGGTAGGTG	N.D.	30
147794	Coding	3	1561	GTAATCTTCATCCCCAGTGC	47	31
147795	Coding	3	1611	TGCTCCATGGTTTGGCCCAT	N.D.	32
147796	Coding	3	1791	GCAGCCAGTCGCTTATCTCC	8	33
147797	Coding	3	2331	GTATAGCCAAAGTGGTCCAC	N.D.	34
147798	Coding	3	2496	CCCAGGAGCTGGAGGTCATG	N.D.	35
147799	Coding	3	2573	TTGAGCCCTTCCTGATGACC	N.D.	36
147800	Coding	3	2811	ATCTGGACCCCACTCCTAGC	N.D.	37
147801	Coding	3	2842	CAGACCCGACTCGTGGAAGA	38	38
147802	Coding	3	3367	GCCCTCAGTAGATTCATCAT	N.D.	39
147803	Coding	3	3611	GCCATGCCACCCTCTTGGAA	N.D.	40
147804	Coding	3	3791	AACCCACGTGCCGGAAAGTC	N.D.	41
147805	Coding	3	3841	ACTCCCAGATGCCTTCTGAA	N.D.	42
147806	Coding	3	4281	ATGTGGTAACGAGCCCGAAG	100	43
147807	Coding	3	4391	GGCGTAGAGACCCATCACAT	25	44
147808	Coding	3	4641	GTGTTAGGATCCCTCTGACA	N.D.	45
147809	Coding	3	5241	CCCAGTGATAGCTCTGTGAG	60	46
147810	Coding	3	5355	ATTTCAGCATATGAGCCCAT	0	47
147811	Coding	3	5691	CCCTGAACCTTAGCAACAGT	N.D.	48
147812	Coding	3	5742	GCTGAAGCCAGCCCAGCGAT	N.D.	49
147813	Coding	3	5891	ACAGCTGCCCAGTATGTTCT	N.D.	50
147814	Coding	3	7087	CCCAATAAGATTTATAACAA	34	51
147815	Coding	3	7731	TGGCCTACCAGAGACAGGTA	45	52
147816	Coding	3	7841	TCATACGTTTAGCCCAATCT	100	53
147817	Coding	3	7901	GCATGGTCCCAAGGATGGTC	0	54
147818	Coding	3	8491	AGTGATGGAAGCTGCGATAC	30	55
147819	Coding	3	9181	ATGAGCATCATGCCTCCCAG	N.D.	56
147820	Coding	3	9931	GAACACATAGCCGAATGCCG	100	57
147821	Coding	3	10263	GTGGTGCCCTCTAATTTGTA	N.D.	58
147822	Coding	3	10631	CCCGAGAAAGAACCGAACCC	N.D.	59
147823	Coding	3	10712	TGCCCTGCAGCTTCACTGAA	19	60
147824	Coding	3	11170	GAAATCCCATAAGCTCTTGT	N.D.	61
147825	Coding	3	12301	AGAAGCTGCCTCTTCTTCCC	72	62
147826	Coding	3	12401	TCAGGGTGAGCCCTGTGTGT	80	63
147827	Coding	3	12471	CTAATGGCCCCTTGATAAAC	13	64
147828	Coding	3	12621	ACGTTATCCTTGAGTCCCTG	12	65
147829	Coding	3	12741	TATATCCCAGGTTTCCCCGG	64	66
147830	Coding	3	12801	ACCTGGGACAGTACCGTCCC	N.D.	67
147831	3'UTR	3	13921	CTGCCTACTGCAAGGCTGGC	0	68
147832	3'UTR	3	13991	AGAGACCTTCCGAGCCCTGG	N.D.	69
147833	3'UTR	3	14101	ATGATACACAATAAAGACTC	25	70

As shown in Table 1, SEQ ID NOs 17, 18, 19, 21, 23, 25, 27, 31, 38, 43, 46, 51, 52, 53, 55, 57, 62, 63 and 66 demonstrated at least 30% inhibition of human apolipoprotein B expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention. As

apolipoprotein B exists in two forms in mammals (ApoB-48 and ApoB-100) which are colinear at the amino terminus, antisense oligonucleotides targeting nucleotides 1-6530 hybridize to both forms, while those targeting nucleotides 6531-14121 are specific to the long form of apolipoprotein B.

Example 16

Antisense inhibition of human apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap-Dose Response Study

In accordance with the present invention, a subset of the antisense oligonuclotides in Example 15 were further investigated in dose-response studies. Treatment doses were 50, 150 and 250 nM. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments and are shown in Table 2.

Table 2

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

	Percent Inhibition		
ISIS #	**50 nM**	**150 nM**	**250 nM**
147788	54	63	72
147806	23	45	28
147816	25	81	65
147820	10	0	73

Example 17

Antisense inhibition of mouse apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides was designed to target different regions of the mouse apolipoprotein B RNA, using published sequence (GenBank accession number M35186, incorporated herein as SEQ ID NO: 10). The oligonucleotides are shown in Table 3. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 3 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on mouse apolipoprotein B mRNA levels in primary mouse hepatocytes by quantitative real-time PCR as described in other examples herein. Primary mouse hepatocytes were treated with 150 nM of the compounds in Table 3. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 3

Inhibition of mouse apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
147475	Coding	10	13	ATTGTATGTGAGAGGTGAGG	79	71
147476	Coding	10	66	GAGGAGATTGGATCTTAAGG	13	72
147477	Coding	10	171	CTTCAAATTGGGACTCTCCT	N.D	73
147478	Coding	10	211	TCCAGGAATTGAGCTTGTGC	78	74
147479	Coding	10	238	TTCAGGACTGGAGGATGAGG	N.D	75
147480	Coding	10	291	TCTCACCCTCATGCTCCATT	54	76
147481	Coding	10	421	TGACTGTCAAGGGTGAGCTG	24	77

147482	Coding	10	461	GTCCAGCCTAGGAACACTCA	59	78
147483	Coding	10	531	ATGTCAATGCCACATGTCCA	N.D	79
147484	Coding	10	581	TTCATCCGAGAAGTTGGGAC	49	80
147485	Coding	10	601	ATTTGGGACGAATGTATGCC	64	81
147486	Coding	10	711	AGTTGAGGAAGCCAGATTCA	N.D	82
147487	Coding	10	964	TTCCCAGTCAGCTTTAGTGG	73	83
147488	Coding	10	1023	AGCTTGCTTGTTGGGCACGG	72	84
147489	Coding	10	1111	CCTATACTGGCTTCTATGTT	5	85
147490	Coding	10	1191	TGAACTCCGTGTAAGGCAAG	N.D	86
147491	Coding	10	1216	GAGAAATCCTTCAGTAAGGG	71	87
147492	Coding	10	1323	CAATGGAATGCTTGTCACTG	68	88
147493	Coding	10	1441	GCTTCATTATAGGAGGTGGT	41	89
147494	Coding	10	1531	ACAACTGGGATAGTGTAGCC	84	90
147495	Coding	10	1631	GTTAGGACCAGGGATTGTGA	0	91
147496	Coding	10	1691	ACCATGGAAAACTGGCAACT	19	92
147497	Coding	10	1721	TGGGAGGAAAAACTTGAATA	N.D	93
147498	Coding	10	1861	TGGGCAACGATATCTGATTG	0	94
147499	Coding	10	1901	CTGCAGGGCGTCAGTGACAA	29	95
147500	Coding	10	1932	GCATCAGACGTGATGTTCCC	N.D	96
147501	Coding	10	2021	CTTGGTTAAACTAATGGTGC	18	97
147502	Coding	10	2071	ATGGGAGCATGGAGGTTGGC	16	98
147503	Coding	10	2141	AATGGATGATGAAACAGTGG	26	99
147504	Coding	10	2201	ATCAATGCCTCCTGTTGCAG	N.D	100
147505	Coding	10	2231	GGAAGTGAGACTTTCTAAGC	76	101
147506	Coding	10	2281	AGGAAGGAACTCTTGATATT	58	102
147507	Coding	10	2321	ATTGGCTTCATTGGCAACAC	81	103
147759	Coding	10	1	AGGTGAGGAAGTTGGAATTC	19	104
147760	Coding	10	121	TTGTTCCCTGAAGTTGTTAC	N.D	105
147761	Coding	10	251	GTTCATGGATTCCTTCAGGA	45	106
147762	Coding	10	281	ATGCTCCATTCTCACATGCT	46	107
147763	Coding	10	338	TGCGACTGTGTCTGATTTCC	34	108
147764	Coding	10	541	GTCCCTGAAGATGTCAATGC	97	109
147765	Coding	10	561	AGGCCCAGTTCCATGACCCT	59	110
147766	Coding	10	761	GGAGCCCACGTGCTGAGATT	59	111
147767	Coding	10	801	CGTCCTTGAGCAGTGCCCGA	5	112
147768	Coding	10	1224	CCCATATGGAGAAATCCTTC	24	113
147769	Coding	10	1581	CATGCCTGGAAGCCAGTGTC	89	114
147770	Coding	10	1741	GTGTTGAATCCCTTGAAATC	67	115
147771	Coding	10	1781	GGTAAAGTTGCCCATGGCTG	68	116
147772	Coding	10	1841	GTTATAAAGTCCAGCATTGG	78	117
147773	Coding	10	1931	CATCAGACGTGATGTTCCCT	85	118
147774	Coding	10	1956	TGGCTAGTTTCAATCCCCTT	84	119
147775	Coding	10	2002	CTGTCATGACTGCCCTTTAC	52	120
147776	Coding	10	2091	GCTTGAAGTTCATTGAGAAT	92	121
147777	Coding	10	2291	TTCCTGAGAAAGGAAGGAAC	N.D	122
147778	Coding	10	2331	TCAGATATACATTGGCTTCA	14	123

As shown in Table 3, SEQ ID Nos 71, 74, 76, 78, 81, 83, 84, 87, 88, 90, 101, 102, 103, 109, 111, 111, 114, 115, 116, 117, 118, 119, 120 and 121 demonstrated at least 50% inhibition of mouse apolipoprotein B expression in this assay and are therefore preferred. The target sites to

which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

Example 18

Antisense inhibition mouse apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap- Dose Response Study

In accordance with the present invention, a subset of the antisense oligonuclotides in Example 17 were further investigated in dose-response studies. Treatment doses were 50, 150 and 300 nM. The compounds were analyzed for their effect on mouse apolipoprotein B mRNA levels in primary hepatocytes cells by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments and are shown in Table 4.

Table 4

Inhibition of mouse apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

	Percent Inhibition		
ISIS #	**50 nM**	**150 nM**	**300 nM**
147483	56	88	89
147764	48	84	90
147769	3	14	28
147776	0	17	44

Example 19

Western blot analysis of apolipoprotein B protein levels

Western blot analysis (immunoblot analysis) was carried out using standard methods. Cells were harvested 16-20 h after oligonucleotide treatment, washed once with

PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels were run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to apolipoprotein B was used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands were visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA) or the ECL+ chemiluminescent detection system (Amersham Biosciences, Piscataway, NJ).

Example 20

Effects of antisense inhibition of apolipoprotein B (ISIS 147764) in C57BL/6 mice: Lean animals vs. High Fat Fed animals.

C57BL/6 mice, a strain reported to be susceptible to hyperlipidemia-induced atherosclerotic plaque formation were used in the following studies to evaluate antisense oligonucleotides as potential lipid lowering compounds in lean versus high fat fed mice.

Male C57BL/6 mice were divided into two matched groups; (1) wild-type control animals (lean animals) and (2) animals receiving a high fat diet (60% kcal fat). Control animals received saline treatment and were maintained on a normal rodent diet. After overnight fasting, mice from each group were dosed intraperitoneally every three days with saline or 50 mg/kg ISIS 147764 (SEQ ID No: 109) for six weeks. At study termination and forty eight hours after the final injections, animals were sacrificed and evaluated for target mRNA levels in liver, cholesterol and triglyceride levels, liver enzyme levels and serum glucose levels.

The results of the comparative studies are shown in Table 5.

Table 5

Effects of ISIS 147764 treatment on apolipoprotein B mRNA, cholesterol, lipid, triglyceride, liver enzyme and glucose levels in lean and high fat mice.

Treatment Group	Percent Change								
			Lipoproteins				Liver Enzymes		
	mRNA	CHOL	VLDL	LDL	HDL	TRIG	AST	ALT	GLUC
Lean-control	-73	-63	No change	-64	-44	-34	Slight decrease	No change	No change
High Fat Group	-87	-67	No change	-87	-65	No change	Slight decrease	Slight increase	-28

It is evident from these data that treatment with ISIS 147764 lowered cholesterol as well as LDL and HDL lipoproteins and serum glucose in both lean and high fat mice and that the effects demonstrated are, in fact, due to the inhibition of apolipoprotein B expression as supported by the decrease in mRNA levels. No significant changes in liver enzyme levels were observed, indicating that the antisense oligonucleotide was not toxic to either treatment group.

Example 21

Effects of antisense inhibition of apolipoprotein B (ISIS 147764) on High Fat Fed Mice; 6 Week Timecourse Study

In accordance with the present invention, a 6-week timecourse study was performed to further investigate the effects of ISIS 147764 on lipid and glucose metabolism in high fat fed mice.

Male C57BL/6 mice (n=8) receiving a high fat diet (60% kcal fat) were evaluated over the course of 6 weeks for the effects of treatment with the antisense oligonucleotide,

ISIS 147764. Control animals received saline treatment (50 mg/kg). A subset of animals received a daily oral dose (20 mg/kg) atorvastatin calcium (Lipitor®, Pfizer Inc.). All mice, except atorvastatin-treated animals, were dosed intraperitoneally every three days (twice a week), after fasting overnight, with 5, 25, 50 mg/kg ISIS 147764 (SEQ ID No: 109) or saline (50 mg/kg) for six weeks. Serum cholesterol and lipoproteins were analyzed at 0, 2 and 6 week interim timepoints. At study termination, animals were sacrificed 48 hours after the final injections and evaluated for levels of target mRNA levels in liver, cholesterol, lipoprotein, triglyceride, liver enzyme (AST and ALT) and serum glucose levels as well as body, liver, spleen and fat pad weights.

Example 22

Effects of antisense inhibition of apolipoprotein B (ISIS 147764) in high fat fed mice- mRNA expression in liver

Male C57BL/6 mice (n=8) receiving a high fat diet (60% kcal fat) were evaluated over the course of 6 weeks for the effects of ISIS 147764 on mRNA expression. Control animals received saline treatment (50 mg/kg). Mice were dosed intraperitoneally every three days (twice a week), after fasting overnight, with 5, 25, 50 mg/kg ISIS 147764 (SEQ ID No: 109) or saline (50 mg/kg) for six weeks. At study termination, animals were sacrificed 48 hours after the final injections and evaluated for levels of target mRNA levels in liver. ISIS 147764 showed a dose-response effect, reducing mRNA levels by 15, 75 and 88% at doses of 5, 25 and 50 mg/kg, respectively.

Liver protein samples collected at the end of the treatment period were subjected to immunoblot analysis

using an antibody directed to mouse apolipoprotein B protein (Gladstone Institute, San Francisco, CA). These data demonstrate that treatment with ISIS 147764 decreases apolipoprotein B protein expression in liver in a dose-dependent manner, in addition to reducing mRNA levels.

Example 23

Effects of antisense inhibition of apolipoprotein B (ISIS 147764) on serum cholesterol and triglyceride levels

Male C57BL/6 mice (n=8) receiving a high fat diet (60% kcal fat) were evaluated over the course of 6 weeks for the effects of ISIS 147764 on serum cholesterol and triglyceride levels. Control animals received saline treatment (50 mg/kg). Mice were dosed intraperitoneally every three days (twice a week), after fasting overnight, with 5, 25, 50 mg/kg ISIS 147764 (SEQ ID No: 109) or saline (50 mg/kg) for six weeks.

Serum cholesterol levels were measured at 0, 2 and 6 weeks and this data is shown in Table 6. Values in the table are expressed as percent inhibition and are normalized to the saline control.

In addition to serum cholesterol, at study termination, animals were sacrificed 48 hours after the final injections and evaluated for triglyceride levels.

Mice treated with ISIS 147764 showed a reduction in both serum cholesterol (240 mg/dL for control animals and 225, 125 and 110 mg/dL for doses of 5, 25, and 50 mg/kg, respectively) and triglycerides (115 mg/dL for control animals and 125, 150 and 85 mg/dL for doses of 5, 25, and 50 mg/kg, respectively) to normal levels by study end. These data were also compared to the effects of atorvastatin calcium at an oral dose of 20 mg/kg which

showed only a minimal decrease in serum cholesterol of 20 percent at study termination.

Table 6

Percent Inhibition of mouse apolipoprotein B cholesterol levels by ISIS 147764

	Percent Inhibition			
time	**Saline**	**5 mg/kg**	**25 mg/kg**	**50 mg/kg**
0 weeks	0	0	0	0
2 weeks	0	5	12	20
6 weeks	0	10	45	55

Example 24

Effects of antisense inhibition of apolipoprotein B (ISIS 147764) on lipoprotein levels

Male C57BL/6 mice (n=8) receiving a high fat diet (60% kcal fat) were evaluated over the course of 6 weeks for the effects of ISIS 147764 on lipoprotein (VLDL, LDL and HDL) levels. Control animals received saline treatment (50 mg/kg). Mice were dosed intraperitoneally every three days (twice a week), after fasting overnight, with 5, 25, 50 mg/kg ISIS 147764 (SEQ ID No: 109) or saline (50 mg/kg) for six weeks.

Lipoprotein levels were measured at 0, 2 and 6 weeks and this data is shown in Table 7. Values in the table are expressed as percent inhibition and are normalized to the saline control. Negative values indicate an observed increase in lipoprotein levels.

These data were also compared to the effects of atorvastatin calcium at a daily oral dose of 20 mg/kg at 0, 2 and 6 weeks.

These data demonstrate that at a dose of 50 mg/kg, ISIS 147764 is capable of lowering all categories of serum

lipoproteins investigated to a greater extent than atorvastatin.

Table 7

Percent Inhibition of mouse apolipoprotein B lipoprotein levels by ISIS 147764 as compared to atorvastatin

		Percent Inhibition				
		Dose				
Lipoprotein	**Time** (weeks)	**Saline**	**5 mg/kg**	**25 mg/kg**	**50 mg/kg**	**atorvastatin (20 mg/kg)**
VLDL	0	0	0	0	0	0
	2	0	25	30	40	15
	6	0	10	-30	15	-5
LDL	0	0	0	0	0	0
	2	0	-30	10	40	10
	6	0	-10	55	90	-10
HDL	0	0	0	0	0	0
	2	0	5	10	10	15
	6	0	10	45	50	20

Example 25

Effects of antisense inhibition of apolipoprotein B (ISIS 147764) on serum AST and ALT levels

Male C57BL/6 mice (n=8) receiving a high fat diet (60% kcal fat) were evaluated over the course of 6 weeks for the effects of ISIS 147764 on liver enzyme (AST and ALT) levels. Increased levels of the liver enzymes ALT and AST indicate toxicity and liver damage. Control animals received saline treatment (50 mg/kg). Mice were dosed intraperitoneally every three days (twice a week), after fasting overnight, with 5, 25, 50 mg/kg ISIS 147764 (SEQ ID No: 109) or saline (50 mg/kg) for six weeks. AST and ALT levels were measured at 6 weeks.

Mice treated with ISIS 147764 showed no significant change in AST levels over the duration of the study compared to saline controls (105, 70 and 80 IU/L for doses

of 5, 25 and 50 mg/kg, respectively compared to 65 IU/L for saline control). Mice treated with atorvastatin at a daily oral dose of 20 mg/kg had AST levels of 85 IU/L.

ALT levels were increased by all treatments with ISIS 147764 over the duration of the study compared to saline controls (50, 70 and 100 IU/L for doses of 5, 25 and 50 mg/kg, respectively compared to 25 IU/L for saline control). Mice treated with atorvastatin at a daily oral dose of 20 mg/kg had AST levels of 40 IU/L.

Example 26

Effects of antisense inhibition of apolipoprotein B (ISIS 147764) on serum glucose levels

Male C57BL/6 mice (n=8) receiving a high fat diet (60% kcal fat) were evaluated over the course of 6 weeks for the effects of ISIS 147764 on serum glucose levels. Control animals received saline treatment (50 mg/kg). Mice were dosed intraperitoneally every three days (twice a week), after fasting overnight, with 5, 25, 50 mg/kg ISIS 147764 (SEQ ID No: 109) or saline (50 mg/kg) for six weeks.

At study termination, animals were sacrificed 48 hours after the final injections and evaluated for serum glucose levels. ISIS 147764 showed a dose-response effect, reducing serum glucose levels to 225, 190 and 180 mg/dL at doses of 5, 25 and 50 mg/kg, respectively compared to the saline control of 300 mg/dL. Mice treated with atorvastatin at a daily oral dose of 20 mg/kg had serum glucose levels of 215 mg/dL. These data demonstrate that ISIS 147764 is capable of reducing serum glucose levels in high fat fed mice.

Example 27

Effects of antisense inhibition of apolipoprotein B (ISIS

147764) on body, spleen, liver and fat pad weight

Male C57BL/6 mice (n=8) receiving a high fat diet (60% kcal fat) were evaluated over the course of 6 weeks for the effects of ISIS 147764 on body, spleen, liver and fat pad weight. Control animals received saline treatment (50 mg/kg). Mice were dosed intraperitoneally every three days (twice a week), after fasting overnight, with 5, 25, 50 mg/kg ISIS 147764 (SEQ ID No: 109) or saline (50 mg/kg) for six weeks.

At study termination, animals were sacrificed 48 hours after the final injections and body, spleen, liver and fat pad weights were measured. These data are shown in Table 8. Values are expressed as percent change in body weight or ogan weight compared to the saline-treated control animals. Data from mice treated with atorvastatin at a daily dose of 20 mg/kg are also shown in the table. Negative values indicated a decrease in weight.

Table 8

Effects of antisense inhibition of mouse apolipoprotein B on body and organ weight

	Percent Change			
	Dose			**Atorvastatin**
Tissue	**5 mg/kg**	**25 mg/kg**	**50 mg/kg**	**20 mg/kg**
Total Body Wt.	5	5	-4	1
Spleen	10	10	46	10
Liver	18	70	80	15
Fat	10	6	-47	7

These data show a decrease in fat over the dosage range of ISIS 147764 counterbalanced by an increase in both spleen and liver weight with increased dose to give an overall decrease in total body weight.

Example 28

Effects of antisense inhibition of apolipoprotein B (ISIS 147764) in B6.129P-Apoe^{tm1Unc} knockout mice: Lean animals vs. High Fat Fed animals.

B6.129P-ApoE^{tm1Unc} knockout mice (herein referred to as ApoE knockout mice) obtained from The Jackson Laboratory (Bar Harbor, ME), are homozygous for the *Apoe^{tm1Unc}* mutation and show a marked increase in total plasma cholesterol levels that are unaffected by age or sex. These animals present with fatty streaks in the proximal aorta at 3 months of age. These lesions increase with age and progress to lesions with less lipid but more elongated cells, typical of a more advanced stage of pre-atherosclerotic lesion.

The mutation in these mice resides in the apolipoprotein E (ApoE) gene. The primary role of the ApoE protein is to transport cholesterol and triglycerides throughout the body. It stabilizes lipoprotein structure, binds to the low density lipoprotein receptor (LDLR) and related proteins, and is present in a subclass of HDLs, providing them the ability to bind to LDLR. ApoE is expressed most abundantly in the liver and brain. Female B6.129P-Apoetm1Unc knockout mice (ApoE knockout mice) were used in the following studies to evaluate antisense oligonucleotides as potential lipid lowering compounds.

Female ApoE knockout mice ranged in age from 5 to 7 weeks and were placed on a normal diet for 2 weeks before study initiation. ApoE knockout mice were then fed *ad libitum* a 60% fat diet, with 0.15% added cholesterol to induce dyslipidemia and obesity. Control animals were maintained on a high-fat diet with no added cholesterol. After overnight fasting, mice from each group were dosed

intraperitoneally every three days with saline, 50 mg/kg of a control antisense oligonucleotide (ISIS 29837; TCGATCTCCTTTTATGCCCG; SEQ ID NO. 124) or 5, 25 or 50 mg/kg ISIS 147764 (SEQ ID No: 109) for six weeks.

The control oligonucleotide is a chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

At study termination and forty eight hours after the final injections, animals were sacrificed and evaluated for target mRNA levels in liver by RT-PCR methods verified by Northern Blot analysis, glucose levels, cholesterol and lipid levels by HPLC separation methods and triglyceride and liver enzyme levels (perfomed by LabCorp Preclinical Services; San Diego, CA). Data from ApoE knockout mice treated with atorvastatin at a daily dose of 20 mg/kg are also shown in the table for comparison.

The results of the comparative studies are shown in Table 9. Data are normalized to saline controls.

Table 9

Effects of ISIS 147764 treatment on apolipoprotein B mRNA, cholesterol, glucose, lipid, triglyceride and liver enzyme levels in ApoE knockout mice.

		Percent Inhibition				
		Dose				
		Control	**5 mg/kg**	**25 mg/kg**	**50 mg/kg**	**atorvastatin (20 mg/kg)**
mRNA		0	2	42	70	10

Glucose	Glucose Levels (mg/dL)					
		225	195	209	191	162
	Cholesterol Levels (mg/dL)					
Cholesterol		1750	1630	1750	1490	938
	Lipoprotein Levels (mg/dL)					
Lipoprotein	HDL	51	49	62	61	42
	LDL	525	475	500	325	250
	VLDL	1190	1111	1194	1113	653
	Liver Enzyme Levels (IU/L)					
Liver Enzymes	AST	55	50	60	85	75
	ALT	56	48	59	87	76

It is evident from these data that treatment with ISIS 147764 lowered glucose and cholesterol as well as all lipoproteins investigated (HDL, LDL and VLDL) in ApoE knockout mice. Further, these decreases correlated with a decrease in both protein and RNA levels of apolipoprotein B, demonstrating an antisense mechanism of action. No significant changes in liver enzyme levels were observed, indicating that the antisense oligonucleotide was not toxic to either treatment group.

Example 29

Antisense inhibition of human apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap: Additional Oligonucleotides

In accordance with the present invention, another series of oligonucleotides was designed to target different regions of the human apolipoprotein B RNA, using published sequence (GenBank accession number NM_000384.1, incorporated herein as SEQ ID NO: 3). The oligonucleotides are shown in Table 10. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds

in Table 10 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments in which HepG2 cells were treated with 150 nM of the compounds in Table 10. If present, "N.D." indicates "no data".

Table 10

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
270985	5'UTR	3	199	TTCCTCTTCGGCCCTGGCGC	75	124
270986	coding	3	299	CTCCACTGGAACTCTCAGCC	0	125
270987	exon: exon junction	3	359	CCTCCAGCTCAACCTTGCAG	0	126
270988	coding	3	429	GGGTTGAAGCCATACACCTC	6	127
270989	exon: exon junction	3	509	CCAGCTTGAGCTCATACCTG	64	128
270990	coding	3	584	CCCTCTTGATGTTCAGGATG	42	129
270991	coding	3	669	GAGCAGTTTCCATACACGGT	21	130
270992	coding	3	699	CCCTTCCTCGTCTTGACGGT	8	131
270993	coding	3	756	TTGAAGCGATCACACTGCCC	69	132
270994	coding	3	799	GCCTTTGATGAGAGCAAGTG	51	133
270995	coding	3	869	TCCTCTTAGCGTCCAGTGTG	40	134
270996	coding	3	1179	CCTCTCAGCTCAGTAACCAG	0	135
270997	coding	3	1279	GCACTGAGGCTGTCCACACT	24	136
270998	coding	3	1419	CGCTGATCCCTCGCCATGTT	1	137

270999	coding	3	1459	GTTGACCGCGTGGCTCAGCG	76	138
271000	coding	3	1499	GCAGCTCCTGGGTCCCTGTA	22	139
271001	coding	3	1859	CCCATGGTAGAATTTGGACA	53	140
271002	exon: exon junction	3	2179	AATCTCGATGAGGTCAGCTG	48	141
271003	coding	3	2299	GACACCATCAGGAACTTGAC	46	142
271004	coding	3	2459	GCTCCTCTCCCAAGATGCGG	10	143
271005	coding	3	2518	GGCACCCATCAGAAGCAGCT	32	144
271006	coding	3	2789	AGTCCGGAATGATGATGCCC	42	145
271007	coding	3	2919	CTGAGCAGCTTGACTGGTCT	26	146
271008	coding	3	3100	CCCGGTCAGCGGATAGTAGG	37	147
271010	exon: exon junction	3	3449	TGTCACAACTTAGGTGGCCC	57	148
271011	coding	3	3919	GTCTGGCAATCCCATGTTCT	51	149
271012	coding	3	4089	CCCACAGACTTGAAGTGGAG	55	150
271013	coding	3	4579	GAACTGCCCATCAATCTTGA	19	151
271014	coding	3	5146	CCCAGAGAGGCCAAGCTCTG	54	152
271015	coding	3	5189	TGTGTTCCCTGAAGCGGCCA	43	153
271016	coding	3	5269	ACCCAGAATCATGGCCTGAT	19	154
271017	coding	3	6049	GGTGCCTGTCTGCTCAGCTG	30	155
271018	coding	3	6520	ATGTGAAACTTGTCTCTCCC	44	156
271019	coding	3	6639	TATGTCTGCAGTTGAGATAG	15	157
271020	coding	3	6859	TTGAATCCAGGATGCAGTAC	35	158
271021	coding	3	7459	GAGTCTCTGAGTCACCTCAC	38	159
271022	coding	3	7819	GATAGAATATTGCTCTGCAA	100	160
271023	coding	3	7861	CCCTTGCTCTACCAATGCTT	44	161
271025	coding	3	8449	TCCATTCCCTATGTCAGCAT	16	162
271026	coding	3	8589	GACTCCTTCAGAGCCAGCGG	39	163
271027	coding	3	8629	CCCATGCTCCGTTCTCAGGT	26	164
271028	coding	3	8829	CGCAGGTCAGCCTGACTAGA	98	165
271030	coding	3	9119	CAGTTAGAACACTGTGGCCC	52	166
271031	coding	3	10159	CAGTGTGATGACACTTGATT	49	167
271032	coding	3	10301	CTGTGGCTAACTTCAATCCC	22	168
271033	coding	3	10349	CAGTACTGTTATGACTACCC	34	169
271034	coding	3	10699	CACTGAAGACCGTGTGCTCT	35	170
271035	coding	3	10811	TCGTACTGTGCTCCCAGAGG	23	171
271036	coding	3	10839	AAGAGGCCCTCTAGCTGTAA	95	172
271037	coding	3	11039	AAGACCCAGAATGAATCCGG	23	173
271038	coding	3	11779	GTCTACCTCAAAGCGTGCAG	29	174
271039	coding	3	11939	TAGAGGCTAACGTACCATCT	4	175
271041	coding	3	12149	CCATATCCATGCCCACGGTG	37	176
271042	coding	3	12265	AGTTTCCTCATCAGATTCCC	57	177
271043	coding	3	12380	CCCAGTGGTACTTGTTGACA	68	178
271044	coding	3	12526	CCCAGTGGTGCCACTGGCTG	22	179
271045	coding	3	12579	GTCAACAGTTCCTGGTACAG	19	180
271046	coding	3	12749	CCCTAGTGTATATCCCAGGT	61	181
271048	coding	3	13009	CTGAAGATTACGTAGCACCT	7	182
271049	coding	3	13299	GTCCAGCCAACTATACTTGG	54	183
271050	coding	3	13779	CCTGGAGCAAGCTTCATGTA	42	184
281586	exon:	3	229	TGGACAGACCAGGCTGACAT	80	185

	exon junction					
281587	coding	3	269	ATGTGTACTTCCGGAGGTGC	77	186
281588	coding	3	389	TCTTCAGGATGAAGCTGCAG	80	187
281589	coding	3	449	TCAGCAAGGCTTTGCCCTCA	90	188
281590	coding	3	529	CTGCTTCCCTTCTGGAATGG	84	189
281591	coding	3	709	TGCCACATTGCCCTTCCTCG	90	190
281592	coding	3	829	GCTGATCAGAGTTGACAAGG	56	191
281593	coding	3	849	TACTGACAGGACTGGCTGCT	93	192
281594	coding	3	889	GATGGCTTCTGCCACATGCT	74	193
281595	coding	3	1059	GATGTGGATTTGGTGCTCTC	76	194
281596	coding	3	1199	TGACTGCTTCATCACTGAGG	77	195
281597	coding	3	1349	GGTAGGTGACCACATCTATC	36	196
281598	coding	3	1390	TCGCAGCTGCTGTGCTGAGG	70	197
281599	exon: exon junction	3	1589	TTCCAATGACCCGCAGAATC	74	198
281600	coding	3	1678	GATCATCAGTGATGGCTTTG	52	199
281601	coding	3	1699	AGCCTGGATGGCAGCTTTCT	83	200
281602	coding	3	1749	GTCTGAAGAAGAACCTCCTG	84	201
281603	coding	3	1829	TATCTGCCTGTGAAGGACTC	82	202
281604	coding	3	1919	CTGAGTTCAAGATATTGGCA	78	203
281605	exon: exon junction	3	2189	CTTCCAAGCCAATCTCGATG	82	204
281606	coding	3	2649	TGCAACTGTAATCCAGCTCC	86	205
281607	exon: exon junction	3	2729	CCAGTTCAGCCTGCATGTTG	84	206
281608	coding	3	2949	GTAGAGACCAAATGTAATGT	62	207
281609	coding	3	3059	CGTTGGAGTAAGCGCCTGAG	70	208
281610	exon: exon junction	3	3118	CAGCTCTAATCTGGTGTCCC	69	209
281611	coding	3	3189	CTGTCCTCTCTCTGGAGCTC	93	210
281612	coding	3	3289	CAAGGTCATACTCTGCCGAT	83	211
281613	coding	3	3488	GTATGGAAATAACACCCTTG	70	212
281614	coding	3	3579	TAAGCTGTAGCAGATGAGTC	63	213
281615	coding	3	4039	TAGATCTCTGGAGGATTTGC	81	214
281616	coding	3	4180	GTCTAGAACACCCAGGAGAG	66	215
281617	coding	3	4299	ACCACAGAGTCAGCCTTCAT	89	216
281618	coding	3	4511	AAGCAGACATCTGTGGTCCC	90	217
281619	coding	3	4660	CTCTCCATTGAGCCGGCCAG	96	218
281620	coding	3	4919	CCTGATATTCAGAACGCAGC	89	219
281621	coding	3	5009	CAGTGCCTAAGATGTCAGCA	53	220
281622	coding	3	5109	AGCACCAGGAGACTACACTT	88	221
281623	coding	3	5212	CCCATCCAGACTGAATTTTG	59	222
281624	coding	3	5562	GGTTCTAGCCGTAGTTTCCC	75	223
281625	coding	3	5589	AGGTTACCAGCCACATGCAG	94	224
281626	coding	3	5839	ATGTGCATCGATGGTCATGG	88	225
281627	coding	3	5869	CCAGAGAGCGAGTTTCCCAT	82	226
281628	coding	3	5979	CTAGACACGAGATGATGACT	81	227
281629	coding	3	6099	TCCAAGTCCTGGCTGTATTC	83	228

281630	coding	3	6144	CGTCCAGTAAGCTCCACGCC	82	229
281631	coding	3	6249	TCAACGGCATCTCTCATCTC	88	230
281632	coding	3	6759	TGATAGTGCTCATCAAGACT	75	231
281633	coding	3	6889	GATTCTGATTTGGTACTTAG	73	232
281634	coding	3	7149	CTCTCGATTAACTCATGGAC	81	233
281635	coding	3	7549	ATACACTGCAACTGTGGCCT	89	234
281636	coding	3	7779	GCAAGAGTCCACCAATCAGA	68	235
281637	coding	3	7929	AGAGCCTGAAGACTGACTTC	74	236
281638	coding	3	8929	TCCCTCATCTGAGAATCTGG	66	237
281640	coding	3	10240	CAGTGCATCAATGACAGATG	87	238
281641	coding	3	10619	CCGAACCCTTGACATCTCCT	72	239
281642	coding	3	10659	GCCTCACTAGCAATAGTTCC	59	240
281643	coding	3	10899	GACATTTGCCATGGAGAGAG	61	241
281644	coding	3	11209	CTGTCTCCTACCAATGCTGG	26	242
281645	exon: exon junction	3	11979	TCTGCACTGAAGTCACGGTG	78	243
281646	coding	3	12249	TCCCGGACCCTCAACTCAGT	76	244
281648	3'UTR	3	13958	GCAGGTCCAGTTCATATGTG	81	245
281649	3'UTR	3	14008	GCCATCCTTCTGAGTTCAGA	76	246
301012	exon: exon junction	3	3249	GCCTCAGTCTGCTTCGCACC	87	247
301013	5'UTR	3	3	CCCCGCAGGTCCCGGTGGGA	82	248
301014	5'UTR	3	6	CAGCCCCGCAGGTCCCGGTG	88	249
301015	5'UTR	3	23	CAACCGAGAAGGGCACTCAG	53	250
301016	5'UTR	3	35	CCTCAGCGGCAGCAACCGAG	62	251
301017	5'UTR	3	36	TCCTCAGCGGCAGCAACCGA	47	252
301018	5'UTR	3	37	CTCCTCAGCGGCAGCAACCG	45	253
301019	5'UTR	3	39	GGCTCCTCAGCGGCAGCAAC	70	254
301020	5'UTR	3	43	GGCGGGCTCCTCAGCGGCAG	85	255
301021	5'UTR	3	116	GGTCCATCGCCAGCTGCGGT	89	256
301022	Start Codon	3	120	GGCGGGTCCATCGCCAGCTG	69	257
301023	Stop Codon	3	13800	TAGAGGATGATAGTAAGTTC	69	258
301024	3'UTR	3	13824	AAATGAAGATTTCTTTTAAA	5	259
301025	3'UTR	3	13854	TATGTGAAAGTTCAATTGGA	76	260
301026	3'UTR	3	13882	ATATAGGCAGTTTGAATTTT	57	261
301027	3'UTR	3	13903	GCTCACTGTATGGTTTTATC	89	262
301028	3'UTR	3	13904	GGCTCACTGTATGGTTTTAT	93	263
301029	3'UTR	3	13908	GGCTGGCTCACTGTATGGTT	90	264
301030	3'UTR	3	13909	AGGCTGGCTCACTGTATGGT	90	265
301031	3'UTR	3	13910	AAGGCTGGCTCACTGTATGG	90	266
301032	3'UTR	3	13917	CTACTGCAAGGCTGGCTCAC	63	267
301033	3'UTR	3	13922	ACTGCCTACTGCAAGGCTGG	77	268
301034	3'UTR	3	13934	TGCTTATAGTCTACTGCCTA	88	269
301035	3'UTR	3	13937	TTCTGCTTATAGTCTACTGC	82	270
301036	3'UTR	3	13964	TTTGGTGCAGGTCCAGTTCA	88	271
301037	3'UTR	3	13968	CAGCTTTGGTGCAGGTCCAG	90	272
301038	3'UTR	3	13970	GCCAGCTTTGGTGCAGGTCC	86	273
301039	3'UTR	3	13974	TGGTGCCAGCTTTGGTGCAG	73	274

301040	3'UTR	3	13978	GCCCTGGTGCCAGCTTTGGT	74	275
301041	3'UTR	3	13997	GAGTTCAGAGACCTTCCGAG	85	276
301042	3'UTR	3	14012	AAATGCCATCCTTCTGAGTT	81	277
301043	3'UTR	3	14014	AAAAATGCCATCCTTCTGAG	81	278
301044	3'UTR	3	14049	AAAATAACTCAGATCCTGAT	76	279
301045	3'UTR	3	14052	AGCAAAATAACTCAGATCCT	90	280
301046	3'UTR	3	14057	AGTTTAGCAAAATAACTCAG	80	281
301047	3'UTR	3	14064	TCCCCCAAGTTTAGCAAAAT	56	282
301048	3'UTR	3	14071	TTCCTCCTCCCCCAAGTTTA	67	283
301217	3'UTR	3	14087	AGACTCCATTTATTTGTTCC	81	284

Example 30

Antisense inhibition of apolipoprotein B - Gene walk

In accordance with the present invention, a "gene walk" was conducted in which another series of oligonucleotides was designed to target the regions of the human apolipoprotein B RNA (GenBank accession number NM_000384.1, incorporated herein as SEQ ID NO: 3) which are near the target site of SEQ ID Nos 224 or 247. The oligonucleotides are shown in Table 11. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 11 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. Treatment doses were 50 nM and 150 nM and are indicated in Table 11. Data are

averages from two experiments. If present, "N.D." indicates "no data".

Table 11

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap - Gene walk

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB 150 nM	% INHIB 50 nM	SEQ ID NO
308589	exon: exon junction	3	3230	CTTCTGCTTGAGTTACAAAC	94	20	285
308590	exon: exon junction	3	3232	ACCTTCTGCTTGAGTTACAA	98	26	286
308591	exon: exon junction	3	3234	GCACCTTCTGCTTGAGTTAC	92	76	287
308592	exon: exon junction	3	3236	TCGCACCTTCTGCTTGAGTT	96	49	288
308593	exon: exon junction	3	3238	CTTCGCACCTTCTGCTTGAG	80	41	289
308594	exon: exon junction	3	3240	TGCTTCGCACCTTCTGCTTG	88	57	290
308595	exon: exon junction	3	3242	TCTGCTTCGCACCTTCTGCT	82	60	291
308596	exon: exon junction	3	3244	AGTCTGCTTCGCACCTTCTG	94	81	292
308597	exon: exon junction	3	3246	TCAGTCTGCTTCGCACCTTC	91	66	293
308598	exon: exon junction	3	3248	CCTCAGTCTGCTTCGCACCT	85	59	294
308599	exon: exon junction	3	3250	AGCCTCAGTCTGCTTCGCAC	94	79	295
308600	coding	3	3252	GTAGCCTCAGTCTGCTTCGC	89	72	296
308601	coding	3	3254	TGGTAGCCTCAGTCTGCTTC	91	63	297
308602	coding	3	3256	CATGGTAGCCTCAGTCTGCT	92	83	298
308603	coding	3	3258	GTCATGGTAGCCTCAGTCTG	97	56	299
308604	coding	3	3260	ATGTCATGGTAGCCTCAGTC	90	73	300
308605	coding	3	3262	GAATGTCATGGTAGCCTCAG	81	50	301
308606	coding	3	3264	TTGAATGTCATGGTAGCCTC	97	54	302

308607	coding	3	3266	ATTTGAATGTCATGGTAGCC	77	9	303
308608	coding	3	3268	ATATTTGAATGTCATGGTAG	85	70	304
308609	coding	3	5582	CAGCCACATGCAGCTTCAGG	96	78	305
308610	coding	3	5584	ACCAGCCACATGCAGCTTCA	90	40	306
308611	coding	3	5586	TTACCAGCCACATGCAGCTT	95	59	307
308612	coding	3	5588	GGTTACCAGCCACATGCAGC	90	75	308
308613	coding	3	5590	TAGGTTACCAGCCACATGCA	87	43	309
308614	coding	3	5592	TTTAGGTTACCAGCCACATG	92	74	310
308615	coding	3	5594	CTTTTAGGTTACCAGCCACA	85	45	311
308616	coding	3	5596	TCCTTTTAGGTTACCAGCCA	81	39	312
308617	coding	3	5598	GCTCCTTTTAGGTTACCAGC	87	77	313
308618	coding	3	5600	AGGCTCCTTTTAGGTTACCA	77	61	314
308619	coding	3	5602	GTAGGCTCCTTTTAGGTTAC	74	69	315
308620	coding	3	5604	TGGTAGGCTCCTTTTAGGTT	88	69	316
308621	coding	3	5606	TTTGGTAGGCTCCTTTTAGG	91	56	317

As shown in Tables 10 and 11, SEQ ID Nos 124, 128, 129, 132, 133, 134, 138, 140, 141, 142, 144, 145, 147, 148, 149, 150, 152, 153, 155, 156, 158, 159, 160, 161, 163, 165, 166, 167, 169, 170, 172, 176, 177, 178, 181, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, and 317 demonstrated at least 30% inhibition of human apolipoprotein B expression in this assay and are therefore preferred. More preferred are SEQ ID Nos 224, 247, and 262. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting

by compounds of the present invention. These preferred target segments are shown in Table 18. The sequences represent the reverse complement of the preferred antisense compounds shown in Tables 10 and 11. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 18 is the species in which each of the preferred target segments was found.

Example 31

Antisense inhibition of human apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap: Targeting GenBank Accession number M14162.1

In accordance with the present invention, another series of oligonucleotides was designed to target different regions of the human apolipoprotein B RNA, using published sequence (GenBank accession number M14162.1, incorporated herein as SEQ ID NO: 318). The oligonucleotides are shown in Table 12. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 12 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. Data are averages from

two experiments in which HepG2 cells were treated with 150 nM of the compounds in Table 12. If present, "N.D." indicates "no data".

Table 12

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
271009	coding	318	3121	GCCTCAGTCTGCTTCGCGCC	75	319
271024	coding	318	8031	GCTCACTGTTCAGCATCTGG	27	320
271029	coding	318	8792	TGAGAATCTGGGCGAGGCCC	N.D.	321
271040	coding	318	11880	GTCCTTCATATTTGCCATCT	0	322
271047	coding	318	12651	CCTCCCTCATGAACATAGTG	32	323
281639	coding	318	9851	GACGTCAGAACCTATGATGG	38	324
281647	coding	318	12561	TGAGTGAGTCAATCAGCTTC	73	325

Example 32

Antisense Inhibition of human apolipoprotein B - Gene walk targeting GenBank Accession number M14162.1

In accordance with the present invention, a "gene walk" was conducted in which another series of oligonucleotides was designed to target the regions of the human apolipoprotein B RNA (GenBank accession number M14162.1, incorporated herein as SEQ ID NO: 318) which are near the target site of SEQ ID NO: 319. The oligonucleotides are shown in Table 13. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 13 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are

composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. Treatment doses were 50 nM and 150 nM and are indicated in Table 13. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 13

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB 150 nM	% INHIB 50 nM	SEQ ID NO
308622	coding	318	3104	GCCTTCTGCTTGAGTTACAA	87	25	326
308623	coding	318	3106	GCGCCTTCTGCTTGAGTTAC	71	62	327
308624	coding	318	3108	TCGCGCCTTCTGCTTGAGTT	89	69	328
308625	coding	318	3110	CTTCGCGCCTTCTGCTTGAG	83	64	329
308626	coding	318	3116	AGTCTGCTTCGCGCCTTCTG	94	38	330
308627	coding	318	3118	TCAGTCTGCTTCGCGCCTTC	89	67	331
308628	coding	318	3120	CCTCAGTCTGCTTCGCGCCT	92	61	332
308629	coding	318	3122	AGCCTCAGTCTGCTTCGCGC	95	77	333

As shown in Tables 12 and 13, SEQ ID Nos 319, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, and 333 demonstrated at least 30% inhibition of human apolipoprotein B expression in this assay and are therefore preferred. More preferred is SEQ ID NO: 319. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target

segments are shown in Table 18. The sequences represent the reverse complement of the preferred antisense compounds shown in Tables 12 and 13. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 18 is the species in which each of the preferred target segments was found.

Example 33

Antisense inhibition of human apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap - Targeting the Genomic sequence

In accordance with the present invention, another series of oligonucleotides was designed to target different regions of the human apolipoprotein B RNA, using published sequence (the complement of nucleotides 39835 to 83279 of the sequence with GenBank accession number NT_022227.9, representing a genomic sequence, incorporated herein as SEQ ID NO: 334). The oligonucleotides are shown in Table 14. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 14 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels in HepG2 cells by quantitative real-time PCR as

described in other examples herein. Data are averages from two experiments in which HepG2 cells were treated with 150 nM of the oligonucleotides in Table 14. If present, "N.D." indicates "no data".

Table 14

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
301049	intron: exon junction	334	904	TCTGTAAGACAGGAGAAAGA	41	335
301050	intron: exon junction	334	913	ATTTCCTCTTCTGTAAGACA	22	336
301051	exon: intron junction	334	952	GATGCCTTACTTGGACAGAC	27	337
301052	intron	334	1945	AGAAATAGCTCTCCCAAGGA	13	338
301053	intron: exon junction	334	1988	GTCGCATCTTCTAACGTGGG	45	339
301054	exon: intron junction	334	2104	TCCTCCATACCTTGCAGTTG	0	340
301055	intron	334	2722	TGGCTCATGTCTACCATATT	49	341
301056	intron	334	2791	CAGTTGAAATGCAGCTAATG	35	342
301057	intron	334	3045	TGCAGACTAGGAGTGAAAGT	30	343
301058	intron	334	3117	AGGAGGATGTCCTTTTATTG	27	344
301059	intron	334	3290	ATCAGAGCACCAAAGGGAAT	12	345
301060	intron: exon junction	334	3381	CCAGCTCAACCTGAGAATTC	17	346
301061	exon: intron junction	334	3527	CATGACTTACCTGGACATGG	52	347
301062	intron	334	3566	CCTCAGCGGACACACACACA	21	348
301063	intron	334	3603	GTCACATCCGTGCCTGGTGC	41	349
301064	intron	334	3864	CAGTGCCTCTGGGACCCCAC	60	350
301065	intron	334	3990	AGCTGCAGTGGCCGATCAGC	50	351
301066	intron	334	4251	GACCTCCCCAGCCACGTGGA	61	352
301067	intron	334	4853	TCTGATCACCATACATTACA	45	353
301068	intron	334	5023	ATTTCCCACTGGGTACTCTC	44	354
301069	intron	334	5055	GGCTGAAGCCCATGCTGACT	44	355

301070	intron	334	5091	GTTGGACAGTCATTCTTTTG	38	356
301071	intron	334	5096	CACTTGTTGGACAGTCATTC	48	357
301072	intron	334	5301	ATTTTAAATTACAGTAGATA	43	358
301073	intron	334	5780	CTGTTCTCCACCCATATCAG	37	359
301074	intron: exon junction	334	6353	GAGCTCATACCTGTCCCAGA	75	360
301075	intron	334	6534	TTCAAGGGCCACTGCTATCA	52	361
301076	intron	334	6641	CCAGTATTTCACGCCAATCC	36	362
301077	intron	334	6661	GGCAGGAGGAACCTCGGGCA	55	363
301078	intron	334	6721	TTTTAAAATTAGACCCAACC	22	364
301079	intron	334	6727	TGACTGTTTTAAAATTAGAC	20	365
301080	intron	334	6788	CCCAGCAAACACAGGTGAAG	25	366
301081	intron	334	7059	GAGTGTGGTCTTGCTAGTGC	46	367
301082	intron	334	7066	CTATGCAGAGTGTGGTCTTG	41	368
301083	intron	334	7189	AGAAGATGCAACCACATGTA	29	369
301084	intron: exon junction	334	7209	ACACGGTATCCTATGGAGGA	49	370
301085	exon: intron junction	334	7365	TGGGACTTACCATGCCTTTG	11	371
301086	intron	334	7702	GGTTTTGCTGCCCTACATCC	30	372
301087	intron	334	7736	ACAAGGAGTCCTTGTGCAGA	40	373
301088	intron	334	8006	ATGTTCACTGAGACAGGCTG	41	374
301089	intron	334	8215	GAAGGTCCATGGTTCATCTG	0	375
301090	intron	334	8239	ATTAGACTGGAAGCATCCTG	39	376
301091	intron	334	8738	GAGATTGGAGACGAGCATTT	35	377
301092	exon: intron junction	334	8881	CATGACCTACTTGTAGGAGA	22	378
301093	intron	334	9208	TGGATTTGGATACACAAGTT	42	379
301094	intron	334	9244	ACTCAATATATATTCATTGA	22	380
301095	intron	334	9545	CAAGGAAGCACACCATGTCA	38	381
301096	intron: exon junction	334	9563	ATACTTATTCCTGGTAACCA	24	382
301097	intron	334	9770	GGTAGCCAGAACACCAGTGT	50	383
301098	intron	334	9776	ACTAGAGGTAGCCAGAACAC	34	384
301099	intron	334	10149	ACCACCTGACATCACAGGTT	24	385
301100	intron	334	10341	TACTGTGACCTATGCCAGGA	55	386
301101	intron	334	10467	GGAGGTGCTACTGTTGACAT	42	387
301102	intron	334	10522	TCCAGACTTGTCTGAGTCTA	47	388
301103	intron	334	10547	TCTAAGAGGTAGAGCTAAAG	7	389
301104	intron	334	10587	CCAGAGATGAGCAACTTAGG	38	390
301105	intron	334	10675	GGCCATGTAAATTGCTCATC	7	391
301106	intron	334	10831	AAAGAAACTATCCTGTATTC	12	392
301107	intron: exon junction	334	10946	TTCTTAGTACCTGGAAGATG	23	393
301108	exon: intron junction	334	11166	CATTAGATACCTGGACACCT	29	394

301109	intron	334	11337	GTTTCATGGAACTCAGCGCA	44	395
301110	intron	334	11457	CTGGAGAGCACCTGCAATAG	35	396
301111	intron	334	11521	TGAAGGGTAGAGAAATCATA	9	397
301112	exon: intron junction	334	12111	GGAAACTCACTTGTTGACCG	25	398
301113	intron	334	12155	AGGTGCAAGATGTTCCTCTG	46	399
301114	intron	334	12162	TGCACAGAGGTGCAAGATGT	16	400
301115	intron	334	12221	CACAAGAGTAAGGAGCAGAG	39	401
301116	intron	334	12987	GATGGATGGTGAGAAATTAC	33	402
301117	intron	334	13025	TAGACAATTGAGACTCAGAA	39	403
301118	intron	334	13057	ATGTGCACACAAGGACATAG	33	404
301119	intron	334	13634	ACATACAAATGGCAATAGGC	33	405
301120	intron	334	13673	TAGGCAAAGGACATGAATAG	30	406
301121	coding	334	14448	TTATGATAGCTACAGAATAA	29	407
301122	exon: intron junction	334	14567	CTGAGATTACCCGCAGAATC	32	408
301123	intron	334	14587	GATGTATGTCATATAAAAGA	26	409
301124	intron: exon junction	334	14680	TTTCCAATGACCTGCATTGA	48	410
301125	intron	334	15444	AGGGATGGTCAATCTGGTAG	57	411
301126	intron	334	15562	GGCTAATAAATAGGGTAGTT	22	412
301127	intron	334	15757	TCCTAGAGCACTATCAAGTA	41	413
301128	intron: exon junction	334	15926	CCTCCTGGTCCTGCAGTCAA	56	414
301129	intron	334	16245	CATTTGCACAAGTGTTTGTT	35	415
301130	intron	334	16363	CTGACACACCATGTTATTAT	10	416
301131	intron: exon junction	334	16399	CTTTTTCAGACTAGATAAGA	0	417
301132	exon: intron junction	334	16637	TCACACTTACCTCGATGAGG	29	418
301133	intron	334	17471	AAGAAAATGGCATCAGGTTT	13	419
301134	intron: exon junction	334	17500	CCAAGCCAATCTGAGAAAGA	25	420
301135	exon: intron junction	334	17677	AAATACACACCTGCTCATGT	20	421
301136	exon: intron junction	334	17683	CTTCACAAATACACACCTGC	20	422
301137	intron	334	18519	AGTGGAAGTTTGGTCTCATT	41	423
301138	intron	334	18532	TTGCTAGCTTCAAAGTGGAA	44	424
301139	intron	334	18586	TCAAGAATAAGCTCCAGATC	41	425
301140	intron	334	18697	GCATACAAGTCACATGAGGT	34	426
301141	intron	334	18969	TACAAGGTGTTTCTTAAGAA	38	427
301142	intron	334	19250	ATGCAGCCAGGATGGGCCTA	54	428
301143	intron:	334	19340	TTACCATATCCTGAGAGTTT	55	429

	exon junction					
301144	intron	334	19802	GCAAAGGTAGAGGAAGGTAT	32	430
301145	intron	334	19813	AAGGACCTTCAGCAAAGGTA	36	431
301146	intron	334	20253	CATAGGAGTACATTTATATA	23	432
301147	intron	334	20398	ATTATGATAAAATCAATTTT	19	433
301148	intron	334	20567	AGAAATTTCACTAGATAGAT	31	434
301149	intron	334	20647	AGCATATTTTGATGAGCTGA	44	435
301150	intron	334	20660	GAAAGGAAGGACTAGCATAT	39	436
301151	intron: exon junction	334	20772	CCTCTCCAATCTGTAGACCC	28	437
301152	intron	334	21316	CTGGATAACTCAGACCTTTG	40	438
301153	intron	334	21407	AGTCAGAAAACAACCTATTC	11	439
301154	intron: exon junction	334	21422	CAGCCTGCATCTATAAGTCA	31	440
301155	exon: intron junction	334	21634	AAAGAATTACCCTCCACTGA	33	441
301156	intron	334	21664	TCTTTCAAACTGGCTAGGCA	39	442
301157	intron	334	21700	GCCTGGCAAAATTCTGCAGG	37	443
301158	intron	334	22032	CTACCTCAAATCAATATGTT	28	444
301159	intron	334	22048	TGCTTTACCTACCTAGCTAC	36	445
301160	intron	334	22551	ACCTTGTGTGTCTCACTCAA	49	446
301161	intron	334	22694	ATGCATTCCCTGACTAGCAC	34	447
301162	intron	334	22866	CATCTCTGAGCCCCTTACCA	24	448
301163	intron	334	22903	GCTGGGCATGCTCTCTCCCC	51	449
301164	intron	334	22912	GCTTTCGCAGCTGGGCATGC	55	450
301165	intron	334	23137	ACTCCTTTCTATACCTGGCT	47	451
301166	intron	334	23170	ATTCTGCCTCTTAGAAAGTT	38	452
301167	intron	334	23402	CCAAGCCTCTTTACTGGGCT	29	453
301168	intron	334	23882	CACTCATGACCAGACTAAGA	35	454
301169	intron	334	23911	ACCTCCCAGAAGCCTTCCAT	22	455
301170	intron	334	24184	TTCATATGAAATCTCCTACT	40	456
301171	intron	334	24425	TATTTAATTTACTGAGAAAC	7	457
301172	intron: exon junction	334	24559	TAATGTGTTGCTGGTGAAGA	35	458
301173	exon: intron junction	334	24742	CATCTCTAACCTGGTGTCCC	21	459
301174	intron	334	24800	GTGCCATGCTAGGTGGCCAT	37	460
301175	intron	334	24957	AGCAAATTGGGATCTGTGCT	29	461
301176	intron	334	24991	TCTGGAGGCTCAGAAACATG	57	462
301177	intron	334	25067	TGAAGACAGGGAGCCACCTA	40	463
301178	intron	334	25152	AGGATTCCCAAGACTTTGGA	38	464
301179	intron: exon junction	334	25351	CAGCTCTAATCTAAAGACAT	22	465
301180	exon: intron junction	334	25473	GAATACTCACCTTCTGCTTG	6	466

301181	intron	334	26047	ATCTCTCTGTCCTCATCTTC	28	467
301182	intron	334	26749	CCAACTCCCCCTTTCTTTGT	37	468
301183	intron	334	26841	TCTGGGCCAGGAAGACACGA	68	469
301184	intron	334	27210	TATTGTGTGCTGGGCACTGC	52	470
301185	intron: exon junction	334	27815	TGCTTCGCACCTGGACGAGT	51	471
301186	exon: intron junction	334	28026	CCTTCTTTACCTTAGGTGGC	37	472
301187	intron	334	28145	GCTCTCTCTGCCACTCTGAT	47	473
301188	intron	334	28769	AACTTCTAAAGCCAACATTC	27	474
301189	intron: exon junction	334	28919	TGTGTCACAACTATGGTAAA	63	475
301190	exon: intron junction	334	29095	AGACACATACCATAATGCCA	22	476
301191	intron: exon junction	334	29204	TTCTCTTCATCTGAAAATAC	21	477
301192	intron	334	29440	TGAGGATGTAATTAGCACTT	27	478
301193	intron: exon junction	334	29871	AGCTCATTGCCTACAAAATG	31	479
301194	intron	334	30181	GTTCTCATGTTTACTAATGC	40	480
301195	intron	334	30465	GAATTGAGACAACTTGATTT	26	481
301196	intron: exon junction	334	30931	CCGGCCATCGCTGAAATGAA	54	482
301197	exon: intron junction	334	31305	CATAGCTCACCTTGCACATT	28	483
301198	intron	334	31325	CGGTGCACCCTTTACCTGAG	28	484
301199	intron: exon junction	334	31813	TCTCCAGATCCTAACATAAA	19	485
301200	intron	334	39562	TTGAATGACACTAGATTTTC	37	486
301201	intron	334	39591	AAAATCCATTTTCTTTAAAG	12	487
301202	intron	334	39654	CAGCTCACACTTATTTTAAA	7	488
301203	intron: exon junction	334	39789	GTTCCCAAAACTGTATAGGA	36	489
301204	exon: intron junction	334	39904	AGCTCCATACTGAAGTCCTT	37	490
301205	intron	334	39916	CAATTCAATAAAAGCTCCAT	31	491
301206	intron	334	39938	GTTTTCAAAAGGTATAAGGT	28	492
301207	intron: exon junction	334	40012	TTCCCATTCCCTGAAAGCAG	13	493
301208	exon: intron junction	334	40196	TGGTATTTACCTGAGGGCTG	21	494

301209	intron	334	40412	ATAAATAATAGTGCTGATGG	39	495
301210	intron	334	40483	CTATGGCTGAGCTTGCCTAT	33	496
301211	intron	334	40505	CTCTCTGAAAAATATACCCT	17	497
301212	intron	334	40576	TTGATGTATCTCATCTAGCA	41	498
301213	intron	334	40658	TAGAACCATGTTTGGTCTTC	35	499
301214	intron	334	40935	TTTCTCTTTATCACATGCCC	29	500
301215	intron	334	41066	TATAGTACACTAAAACTTCA	1	501
301216	intron: exon junction	334	41130	CTGGAGAGGACTAAACAGAG	49	502

As shown in Table 14, SEQ ID Nos 335, 339, 341, 342, 343, 347, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 367, 368, 370, 372, 373, 374, 376, 377, 379, 381, 383, 384, 386, 387, 388, 390, 395, 396, 399, 401, 402, 403, 404, 405, 406, 408, 410, 411, 413, 414, 415, 423, 424, 425, 426, 427, 428, 429, 430, 431, 434, 435, 436, 438, 440, 441, 442, 443, 445, 446, 447, 449, 450, 451, 452, 454, 456, 458, 460, 462, 463, 464, 468, 469, 470, 471, 472, 473, 475, 479, 480, 482, 486, 489, 490, 491, 495, 496, 498, 499, and 502 demonstrated at least 30% inhibition of human apolipoprotein B expression in this assay and are therefore preferred. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target segments are shown in Table 18. The sequences represent the reverse complement of the preferred antisense compounds shown in Table 14. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 18 is the species in which each of the preferred target segments was found.

Example 34

Antisense inhibition of human apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap - Targeting GenBank accession number AI249040.1

In accordance with the present invention, another series of oligonucleotides was designed to target different regions of the human apolipoprotein B RNA, using published sequence (the complement of the sequence with GenBank accession number AI249040.1, incorporated herein as SEQ ID NO: 503). The oligonucleotides are shown in Table 15. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 15 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels in HepG2 cells by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments in which HepG2 cells were treated with 150 nM of the oligonucleotides in Table 15. If present, "N.D." indicates "no data".

Table 15

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE

wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
301218	3'UTR	503	484	ACATTTTATCAATGCCCTCG	23	504
301219	3'UTR	503	490	GCCAGAACATTTTATCAATG	35	505
301220	3'UTR	503	504	AGAGGTTTTGCTGTGCCAGA	51	506
301221	3'UTR	503	506	CTAGAGGTTTTGCTGTGCCA	61	507
301222	3'UTR	503	507	TCTAGAGGTTTTGCTGTGCC	14	508
301223	3'UTR	503	522	AATCACACTATGTGTTCTAG	26	509
301224	3'UTR	503	523	AAATCACACTATGTGTTCTA	33	510
301225	3'UTR	503	524	TAAATCACACTATGTGTTCT	3	511
301226	3'UTR	503	526	CTTAAATCACACTATGTGTT	39	512
301227	3'UTR	503	536	TATTCTGTTACTTAAATCAC	23	513

As shown in Table 15, SEQ ID Nos 505, 506, 507, 510, and 512 demonstrated at least 30% inhibition of human apolipoprotein B expression in this assay and are therefore preferred. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target segments are shown in Table 18. The sequences represent the reverse complement of the preferred antisense compounds shown in Table 15. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 18 is the species in which each of the preferred target segments was found.

Example 35

Antisense inhibition of human apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap - Variation in position of the gap

In accordance with the present invention, a series of antisense compounds was designed to target different regions of the human apolipoprotein B RNA, using published

sequences (GenBank accession number NM_000384.1, incorporated herein as SEQ ID NO: 3). The compounds are shown in Table 16. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the compound binds. All compounds in Table 16 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length. The "gap" region consists of 2'-deoxynucleotides, which is flanked on one or both sides (5' and 3' directions) by "wings" composed of 2'-methoxyethyl (2'-MOE)nucleotides. The number of 2'-MOE nucleotides on either side of the gap varies such that the total number of 2'-MOE nucleotides always equals 10 and the total length of the chimeric oligonucleotide is 20 nucleotides. The exact structure of each oligonucleotide is designated in Table 16 as the "gap structure" and the 2'-deoxynucleotides are in bold type. A designation of 8~10~2, for instance, indicates that the first (5'-most) 8 nucleotides and the last (3'-most) 2 nucleotides are 2'-MOE nucleotides and the 10 nucleotides in the gap are 2'-deoxynucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human apolipoprotein B mRNA levels by quantitative real-time PCR as described in other examples herein. Data, shown in Table 16, are averages from three experiments in which HepG2 cells were treated with the antisense oligonucleotides of the present invention at doses of 50 nM and 150 nM. If present, "N.D." indicates "no data".

Table 16

Inhibition of human apolipoprotein B mRNA levels by

chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a variable deoxy gap

ISIS #	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB 150 nM	% INHIB 50 nM	gap structure	SEQ ID NO
308631	3	5589	**AGGTTACCAG**CCACATGCAG	94	74	0~10~10	224
308632	3	3249	**GCCTCAGTCT**GCTTCGCACC	97	41	0~10~10	247
308634	3	5589	AGGTTACCAG**CCACATGCAG**	67	45	10~10~0	224
308635	3	3249	GCCTCAGTCT**GCTTCGCACC**	93	69	10~10~0	247
308637	3	5589	A**GGTTACCAGC**CACATGCAG	95	79	1~10~9	224
308638	3	3249	G**CCTCAGTCTG**CTTCGCACC	94	91	1~10~9	247
308640	3	5589	AG**GTTACCAGCC**ACATGCAG	96	76	2~10~8	224
308641	3	3249	GC**CTCAGTCTGC**TTCGCACC	89	77	2~10~8	247
308643	3	5589	AGG**TTACCAGCCA**CATGCAG	96	56	3~10~7	224
308644	3	3249	GCC**TCAGTCTGCT**TCGCACC	93	71	3~10~7	247
308646	3	5589	AGGT**TACCAGCCAC**ATGCAG	76	50	4~10~6	224
308647	3	3249	GCCT**CAGTCTGCTT**CGCACC	86	53	4~10~6	247
308649	3	5589	AGGTTA**CCAGCCACAT**GCAG	91	68	6~10~4	224
308650	3	3249	GCCTCA**GTCTGCTTCG**CACC	94	74	6~10~4	247
308652	3	5589	AGGTTAC**CAGCCACATG**CAG	95	73	7~10~3	224
308653	3	3249	GCCTCAG**TCTGCTTCGC**ACC	89	73	7~10~3	247
308655	3	5589	AGGTTACC**AGCCACATGC**AG	83	84	8~10~2	224
308656	3	3249	GCCTCAGT**CTGCTTCGCA**CC	97	37	8~10~2	247
308658	3	5589	AGGTTACCA**GCCACATGCA**G	78	86	9~10~1	224
308659	3	3249	GCCTCAGTC**TGCTTCGCAC**C	93	70	9~10~1	247
308660	3	3254	TG**GTAGCCTCAG**TCTGCTTC	92	72	2~10~8	514
308662	3	3254	TGGTAGCC**TCAGTCTGCT**TC	83	76	8~10~2	514

As shown in Table 16, SEQ ID Nos 224, 247, and 514 demonstrated at least 30% inhibition of human apolipoprotein B expression in this assay at both doses. These data suggest that the oligonucleotides are effective with a number of variations in the gap placement. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target segments are shown in Table 18. The sequences represent the reverse complement of the preferred antisense compounds shown in Table 16. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic

acid to which the oligonucleotide binds. Also shown in Table 18 is the species in which each of the preferred target segments was found.

Example 36

Antisense inhibition of human apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap - Variation in position of the gap of SEQ ID Nos: 319 and 515

In accordance with the present invention, a series of antisense compounds was designed based on SEQ ID Nos 319 and 515, with variations in the gap structure. The compounds are shown in Table 17. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the compound binds. All compounds in Table 17 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length. The "gap" region consists of 2'-deoxynucleotides, which is flanked on one or both sides (5' and 3' directions) by "wings" composed of 2'-methoxyethyl (2'-MOE)nucleotides. The number of 2'-MOE nucleotides on either side of the gap varies such that the total number of 2'-MOE nucleotides always equals 10 and the total length of the chimeric oligonucleotide is 20 nucleotides. The exact structure of each oligonucleotide is designated in Table 17 as the "gap structure" and the 2'-deoxynucleotides are in bold type. A designation of 8~10~2, for instance, indicates that the first (5'-most) 8 nucleotides and the last (3'-most) 2 nucleotides are 2'-MOE nucleotides and the 10 nucleotides in the gap are 2'-deoxynucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were

analyzed for their effect on human apolipoprotein B mRNA levels by quantitative real-time PCR as described in other examples herein. Data, shown in Table 17, are averages from three experiments in which HepG2 cells were treated with the antisense oligonucleotides of the present invention at doses of 50 nM and 150 nM. If present, "N.D." indicates "no data".

Table 17

Inhibition of human apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a variable deoxy gap

ISIS #	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB 150 nM	% INHIB 50 nM	gap structure	SEQ ID NO
308630	318	3121	**GCCTCAGTCT**GCTTCGCGCC	89	69	0~10~10	319
308633	318	3121	GCCTCAGTCT**GCTTCGCGCC**	83	66	10~10~0	319
308636	318	3121	G**CCTCAGTCTG**CTTCGCGCC	91	81	1~10~9	319
308639	318	3121	GC**CTCAGTCTGC**TTCGCGCC	94	86	2~10~8	319
308642	318	3121	GCC**TCAGTCTGCT**TCGCGCC	95	85	3~10~7	319
308645	318	3121	GCCT**CAGTCTGCTT**CGCGCC	98	57	4~10~6	319
308648	318	3121	GCCTCA**GTCTGCTTCG**CGCC	89	78	6~10~4	319
308651	318	3121	GCCTCAG**TCTGCTTCGC**GCC	88	87	7~10~3	319
308654	318	3121	GCCTCAGT**CTGCTTCGCG**CC	90	81	8~10~2	319
308657	318	3121	GCCTCAGTC**TGCTTCGCGC**C	78	61	9~10~1	319
308661	318	3116	AG**TCTGCTTCGC**GCCTTCTG	91	70	2~10~8	515
308663	318	3116	AGTCTGCTT**CGCGCCTTC**TG	84	44	8~10~2	515

As shown in Table 17, SEQ ID Nos 319 and 515 demonstrated at least 44% inhibition of human apolipoprotein B expression in this assay for either dose. These data suggest that the compounds are effective with a number of variations in gap placement. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target segments are

shown in Table 18. The sequences represent the reverse complement of the preferred antisense compounds shown in Table 17. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 18 is the species in which each of the preferred target segments was found.

Table 18

Sequence and position of preferred target segments identified in apolipoprotein B.

SITE ID	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	REV COMP OF SEQ ID NO	ACTIVE IN	SEQ ID NO
187342	3	199	GCGCCAGGGCCGAAGAGGAA	124	*H. sapiens*	516
187346	3	509	CAGGTATGAGCTCAAGCTGG	128	*H. sapiens*	517
187347	3	584	CATCCTGAACATCAAGAGGG	129	*H. sapiens*	518
187350	3	756	GGGCAGTGTGATCGCTTCAA	132	*H. sapiens*	519
187351	3	799	CACTTGCTCTCATCAAAGGC	133	*H. sapiens*	520
187352	3	869	CACACTGGACGCTAAGAGGA	134	*H. sapiens*	521
187356	3	1459	CGCTGAGCCACGCGGTCAAC	138	*H. sapiens*	522
187358	3	1859	TGTCCAAATTCTACCATGGG	140	*H. sapiens*	523
187359	3	2179	CAGCTGACCTCATCGAGATT	141	*H. sapiens*	524
187360	3	2299	GTCAAGTTCCTGATGGTGTC	142	*H. sapiens*	525
187362	3	2518	AGCTGCTTCTGATGGGTGCC	144	*H. sapiens*	526
187363	3	2789	GGGCATCATCATTCCGGACT	145	*H. sapiens*	527
187365	3	3100	CCTACTATCCGCTGACCGGG	147	*H. sapiens*	528
187367	3	3449	GGGCCACCTAAGTTGTGACA	148	*H. sapiens*	529
187368	3	3919	AGAACATGGGATTGCCAGAC	149	*H. sapiens*	530
187369	3	4089	CTCCACTTCAAGTCTGTGGG	150	*H. sapiens*	531
187371	3	5146	CAGAGCTTGGCCTCTCTGGG	152	*H. sapiens*	532
187372	3	5189	TGGCCGCTTCAGGGAACACA	153	*H. sapiens*	533
187374	3	6049	CAGCTGAGCAGACAGGCACC	155	*H. sapiens*	534
187375	3	6520	GGGAGAGACAAGTTTCACAT	156	*H. sapiens*	535
187377	3	6859	GTACTGCATCCTGGATTCAA	158	*H. sapiens*	536
187378	3	7459	GTGAGGTGACTCAGAGACTC	159	*H. sapiens*	537
187379	3	7819	TTGCAGAGCAATATTCTATC	160	*H. sapiens*	538
187380	3	7861	AAGCATTGGTAGAGCAAGGG	161	*H. sapiens*	539
187383	3	8589	CCGCTGGCTCTGAAGGAGTC	163	*H. sapiens*	540
187385	3	8829	TCTAGTCAGGCTGACCTGCG	165	*H. sapiens*	541
187387	3	9119	GGGCCACAGTGTTCTAACTG	166	*H. sapiens*	542
187388	3	10159	AATCAAGTGTCATCACACTG	167	*H. sapiens*	543
187390	3	10349	GGGTAGTCATAACAGTACTG	169	*H. sapiens*	544

187391	3	10699	AGAGCACACGGTCTTCAGTG	170	*H. sapiens*	545
187393	3	10839	TTACAGCTAGAGGGCCTCTT	172	*H. sapiens*	546
187398	3	12149	CACCGTGGGCATGGATATGG	176	*H. sapiens*	547
187399	3	12265	GGGAATCTGATGAGGAAACT	177	*H. sapiens*	548
187400	3	12380	TGTCAACAAGTACCACTGGG	178	*H. sapiens*	549
187403	3	12749	ACCTGGGATATACACTAGGG	181	*H. sapiens*	550
187406	3	13299	CCAAGTATAGTTGGCTGGAC	183	*H. sapiens*	551
187407	3	13779	TACATGAAGCTTGCTCCAGG	184	*H. sapiens*	552
197724	3	229	ATGTCAGCCTGGTCTGTCCA	185	*H. sapiens*	553
197725	3	269	GCACCTCCGGAAGTACACAT	186	*H. sapiens*	554
197726	3	389	CTGCAGCTTCATCCTGAAGA	187	*H. sapiens*	555
197727	3	449	TGAGGGCAAAGCCTTGCTGA	188	*H. sapiens*	556
197728	3	529	CCATTCCAGAAGGGAAGCAG	189	*H. sapiens*	557
197729	3	709	CGAGGAAGGGCAATGTGGCA	190	*H. sapiens*	558
197730	3	829	CCTTGTCAACTCTGATCAGC	191	*H. sapiens*	559
197731	3	849	AGCAGCCAGTCCTGTCAGTA	192	*H. sapiens*	560
197732	3	889	AGCATGTGGCAGAAGCCATC	193	*H. sapiens*	561
197733	3	1059	GAGAGCACCAAATCCACATC	194	*H. sapiens*	562
197734	3	1199	CCTCAGTGATGAAGCAGTCA	195	*H. sapiens*	563
197735	3	1349	GATAGATGTGGTCACCTACC	196	*H. sapiens*	564
197736	3	1390	CCTCAGCACAGCAGCTGCGA	197	*H. sapiens*	565
197737	3	1589	GATTCTGCGGGTCATTGGAA	198	*H. sapiens*	566
197738	3	1678	CAAAGCCATCACTGATGATC	199	*H. sapiens*	567
197739	3	1699	AGAAAGCTGCCATCCAGGCT	200	*H. sapiens*	568
197740	3	1749	CAGGAGGTTCTTCTTCAGAC	201	*H. sapiens*	569
197741	3	1829	GAGTCCTTCACAGGCAGATA	202	*H. sapiens*	570
197742	3	1919	TGCCAATATCTTGAACTCAG	203	*H. sapiens*	571
197743	3	2189	CATCGAGATTGGCTTGGAAG	204	*H. sapiens*	572
197744	3	2649	GGAGCTGGATTACAGTTGCA	205	*H. sapiens*	573
197745	3	2729	CAACATGCAGGCTGAACTGG	206	*H. sapiens*	574
197746	3	2949	ACATTACATTTGGTCTCTAC	207	*H. sapiens*	575
197747	3	3059	CTCAGGCGCTTACTCCAACG	208	*H. sapiens*	576
197748	3	3118	GGGACACCAGATTAGAGCTG	209	*H. sapiens*	577
197749	3	3189	GAGCTCCAGAGAGAGGACAG	210	*H. sapiens*	578
197750	3	3289	ATCGGCAGAGTATGACCTTG	211	*H. sapiens*	579
197751	3	3488	CAAGGGTGTTATTTCCATAC	212	*H. sapiens*	580
197752	3	3579	GACTCATCTGCTACAGCTTA	213	*H. sapiens*	581
197753	3	4039	GCAAATCCTCCAGAGATCTA	214	*H. sapiens*	582
197754	3	4180	CTCTCCTGGGTGTTCTAGAC	215	*H. sapiens*	583
197755	3	4299	ATGAAGGCTGACTCTGTGGT	216	*H. sapiens*	584
197756	3	4511	GGGACCACAGATGTCTGCTT	217	*H. sapiens*	585
197757	3	4660	CTGGCCGGCTCAATGGAGAG	218	*H. sapiens*	586
197758	3	4919	GCTGCGTTCTGAATATCAGG	219	*H. sapiens*	587
197759	3	5009	TGCTGACATCTTAGGCACTG	220	*H. sapiens*	588
197760	3	5109	AAGTGTAGTCTCCTGGTGCT	221	*H. sapiens*	589
197761	3	5212	CAAAATTCAGTCTGGATGGG	222	*H. sapiens*	590
197762	3	5562	GGGAAACTACGGCTAGAACC	223	*H. sapiens*	591
197763	3	5589	CTGCATGTGGCTGGTAACCT	224	*H. sapiens*	592

197764	3	5839	CCATGACCATCGATGCACAT	225	*H. sapiens*	593
197765	3	5869	ATGGGAAACTCGCTCTCTGG	226	*H. sapiens*	594
197766	3	5979	AGTCATCATCTCGTGTCTAG	227	*H. sapiens*	595
197767	3	6099	GAATACAGCCAGGACTTGGA	228	*H. sapiens*	596
197768	3	6144	GGCGTGGAGCTTACTGGACG	229	*H. sapiens*	597
197769	3	6249	GAGATGAGAGATGCCGTTGA	230	*H. sapiens*	598
197770	3	6759	AGTCTTGATGAGCACTATCA	231	*H. sapiens*	599
197771	3	6889	CTAAGTACCAAATCAGAATC	232	*H. sapiens*	600
197772	3	7149	GTCCATGAGTTAATCGAGAG	233	*H. sapiens*	601
197773	3	7549	AGGCCACAGTTGCAGTGTAT	234	*H. sapiens*	602
197774	3	7779	TCTGATTGGTGGACTCTTGC	235	*H. sapiens*	603
197775	3	7929	GAAGTCAGTCTTCAGGCTCT	236	*H. sapiens*	604
197776	3	8929	CCAGATTCTCAGATGAGGGA	237	*H. sapiens*	605
197778	3	10240	CATCTGTCATTGATGCACTG	238	*H. sapiens*	606
197779	3	10619	AGGAGATGTCAAGGGTTCGG	239	*H. sapiens*	607
197780	3	10659	GGAACTATTGCTAGTGAGGC	240	*H. sapiens*	608
197781	3	10899	CTCTCTCCATGGCAAATGTC	241	*H. sapiens*	609
197783	3	11979	CACCGTGACTTCAGTGCAGA	243	*H. sapiens*	610
197784	3	12249	ACTGAGTTGAGGGTCCGGGA	244	*H. sapiens*	611
197786	3	13958	CACATATGAACTGGACCTGC	245	*H. sapiens*	612
197787	3	14008	TCTGAACTCAGAAGGATGGC	246	*H. sapiens*	613
216825	3	3249	GGTGCGAAGCAGACTGAGGC	247	*H. sapiens*	614
216826	3	3	TCCCACCGGGACCTGCGGGG	248	*H. sapiens*	615
216827	3	6	CACCGGGACCTGCGGGGCTG	249	*H. sapiens*	616
216828	3	23	CTGAGTGCCCTTCTCGGTTG	250	*H. sapiens*	617
216829	3	35	CTCGGTTGCTGCCGCTGAGG	251	*H. sapiens*	618
216830	3	36	TCGGTTGCTGCCGCTGAGGA	252	*H. sapiens*	619
216831	3	37	CGGTTGCTGCCGCTGAGGAG	253	*H. sapiens*	620
216832	3	39	GTTGCTGCCGCTGAGGAGCC	254	*H. sapiens*	621
216833	3	43	CTGCCGCTGAGGAGCCCGCC	255	*H. sapiens*	622
216834	3	116	ACCGCAGCTGGCGATGGACC	256	*H. sapiens*	623
216835	3	120	CAGCTGGCGATGGACCCGCC	257	*H. sapiens*	624
216836	3	13800	GAACTTACTATCATCCTCTA	258	*H. sapiens*	625
216838	3	13854	TCCAATTGAACTTTCACATA	260	*H. sapiens*	626
216839	3	13882	AAAATTCAAACTGCCTATAT	261	*H. sapiens*	627
216840	3	13903	GATAAAACCATACAGTGAGC	262	*H. sapiens*	628
216841	3	13904	ATAAAACCATACAGTGAGCC	263	*H. sapiens*	629
216842	3	13908	AACCATACAGTGAGCCAGCC	264	*H. sapiens*	630
216843	3	13909	ACCATACAGTGAGCCAGCCT	265	*H. sapiens*	631
216844	3	13910	CCATACAGTGAGCCAGCCTT	266	*H. sapiens*	632
216845	3	13917	GTGAGCCAGCCTTGCAGTAG	267	*H. sapiens*	633
216846	3	13922	CCAGCCTTGCAGTAGGCAGT	268	*H. sapiens*	634
216847	3	13934	TAGGCAGTAGACTATAAGCA	269	*H. sapiens*	635
216848	3	13937	GCAGTAGACTATAAGCAGAA	270	*H. sapiens*	636
216849	3	13964	TGAACTGGACCTGCACCAAA	271	*H. sapiens*	637
216850	3	13968	CTGGACCTGCACCAAAGCTG	272	*H. sapiens*	638
216851	3	13970	GGACCTGCACCAAAGCTGGC	273	*H. sapiens*	639
216852	3	13974	CTGCACCAAAGCTGGCACCA	274	*H. sapiens*	640

216853	3	13978	ACCAAAGCTGGCACCAGGGC	275	*H. sapiens*	641
216854	3	13997	CTCGGAAGGTCTCTGAACTC	276	*H. sapiens*	642
216855	3	14012	AACTCAGAAGGATGGCATTT	277	*H. sapiens*	643
216856	3	14014	CTCAGAAGGATGGCATTTTT	278	*H. sapiens*	644
216857	3	14049	ATCAGGATCTGAGTTATTTT	279	*H. sapiens*	645
216858	3	14052	AGGATCTGAGTTATTTTGCT	280	*H. sapiens*	646
216859	3	14057	CTGAGTTATTTTGCTAAACT	281	*H. sapiens*	647
216860	3	14064	ATTTTGCTAAACTTGGGGGA	282	*H. sapiens*	648
216861	3	14071	TAAACTTGGGGGAGGAGGAA	283	*H. sapiens*	649
217030	3	14087	GGAACAAATAAATGGAGTCT	284	*H. sapiens*	650
224316	3	3230	GTTTGTAACTCAAGCAGAAG	285	*H. sapiens*	651
224317	3	3232	TTGTAACTCAAGCAGAAGGT	286	*H. sapiens*	652
224318	3	3234	GTAACTCAAGCAGAAGGTGC	287	*H. sapiens*	653
224319	3	3236	AACTCAAGCAGAAGGTGCGA	288	*H. sapiens*	654
224320	3	3238	CTCAAGCAGAAGGTGCGAAG	289	*H. sapiens*	655
224321	3	3240	CAAGCAGAAGGTGCGAAGCA	290	*H. sapiens*	656
224322	3	3242	AGCAGAAGGTGCGAAGCAGA	291	*H. sapiens*	657
224323	3	3244	CAGAAGGTGCGAAGCAGACT	292	*H. sapiens*	658
224324	3	3246	GAAGGTGCGAAGCAGACTGA	293	*H. sapiens*	659
224325	3	3248	AGGTGCGAAGCAGACTGAGG	294	*H. sapiens*	660
224326	3	3250	GTGCGAAGCAGACTGAGGCT	295	*H. sapiens*	661
224327	3	3252	GCGAAGCAGACTGAGGCTAC	296	*H. sapiens*	662
224328	3	3254	GAAGCAGACTGAGGCTACCA	297	*H. sapiens*	663
224329	3	3256	AGCAGACTGAGGCTACCATG	298	*H. sapiens*	664
224330	3	3258	CAGACTGAGGCTACCATGAC	299	*H. sapiens*	665
224331	3	3260	GACTGAGGCTACCATGACAT	300	*H. sapiens*	666
224332	3	3262	CTGAGGCTACCATGACATTC	301	*H. sapiens*	667
224333	3	3264	GAGGCTACCATGACATTCAA	302	*H. sapiens*	668
224334	3	3266	GGCTACCATGACATTCAAAT	303	*H. sapiens*	669
224335	3	3268	CTACCATGACATTCAAATAT	304	*H. sapiens*	670
224336	3	5582	CCTGAAGCTGCATGTGGCTG	305	*H. sapiens*	671
224337	3	5584	TGAAGCTGCATGTGGCTGGT	306	*H. sapiens*	672
224338	3	5586	AAGCTGCATGTGGCTGGTAA	307	*H. sapiens*	673
224339	3	5588	GCTGCATGTGGCTGGTAACC	308	*H. sapiens*	674
224340	3	5590	TGCATGTGGCTGGTAACCTA	309	*H. sapiens*	675
224341	3	5592	CATGTGGCTGGTAACCTAAA	310	*H. sapiens*	676
224342	3	5594	TGTGGCTGGTAACCTAAAAG	311	*H. sapiens*	677
224343	3	5596	TGGCTGGTAACCTAAAAGGA	312	*H. sapiens*	678
224344	3	5598	GCTGGTAACCTAAAAGGAGC	313	*H. sapiens*	679
224345	3	5600	TGGTAACCTAAAAGGAGCCT	314	*H. sapiens*	680
224346	3	5602	GTAACCTAAAAGGAGCCTAC	315	*H. sapiens*	681
224347	3	5604	AACCTAAAAGGAGCCTACCA	316	*H. sapiens*	682
224348	3	5606	CCTAAAAGGAGCCTACCAAA	317	*H. sapiens*	683
187366	318	3121	GGCGCGAAGCAGACTGAGGC	319	*H. sapiens*	684
187404	318	12651	CACTATGTTCATGAGGGAGG	323	*H. sapiens*	685
197777	318	9851	CCATCATAGGTTCTGACGTC	324	*H. sapiens*	686
197785	318	12561	GAAGCTGATTGACTCACTCA	325	*H. sapiens*	687
224349	318	3104	TTGTAACTCAAGCAGAAGGC	326	*H. sapiens*	688

224350	318	3106	GTAACTCAAGCAGAAGGCGC	327	H. sapiens	689
224351	318	3108	AACTCAAGCAGAAGGCGCGA	328	H. sapiens	690
224352	318	3110	CTCAAGCAGAAGGCGCGAAG	329	H. sapiens	691
224353	318	3116	CAGAAGGCGCGAAGCAGACT	330	H. sapiens	692
224354	318	3118	GAAGGCGCGAAGCAGACTGA	331	H. sapiens	693
224355	318	3120	AGGCGCGAAGCAGACTGAGG	332	H. sapiens	694
224356	318	3122	GCGCGAAGCAGACTGAGGCT	333	H. sapiens	695
224328	3	3254	GAAGCAGACTGAGGCTACCA	514	H. sapiens	696
224353	318	3116	CAGAAGGCGCGAAGCAGACT	515	H. sapiens	697
216862	334	904	TCTTTCTCCTGTCTTACAGA	335	H. sapiens	698
216866	334	1988	CCCACGTTAGAAGATGCGAC	339	H. sapiens	699
216868	334	2722	AATATGGTAGACATGAGCCA	341	H. sapiens	700
216869	334	2791	CATTAGCTGCATTTCAACTG	342	H. sapiens	701
216870	334	3045	ACTTTCACTCCTAGTCTGCA	343	H. sapiens	702
216874	334	3527	CCATGTCCAGGTAAGTCATG	347	H. sapiens	703
216876	334	3603	GCACCAGGCACGGATGTGAC	349	H. sapiens	704
216877	334	3864	GTGGGGTCCCAGAGGCACTG	350	H. sapiens	705
216878	334	3990	GCTGATCGGCCACTGCAGCT	351	H. sapiens	706
216879	334	4251	TCCACGTGGCTGGGGAGGTC	352	H. sapiens	707
216880	334	4853	TGTAATGTATGGTGATCAGA	353	H. sapiens	708
216881	334	5023	GAGAGTACCCAGTGGGAAAT	354	H. sapiens	709
216882	334	5055	AGTCAGCATGGGCTTCAGCC	355	H. sapiens	710
216883	334	5091	CAAAAGAATGACTGTCCAAC	356	H. sapiens	711
216884	334	5096	GAATGACTGTCCAACAAGTG	357	H. sapiens	712
216885	334	5301	TATCTACTGTAATTTAAAAT	358	H. sapiens	713
216886	334	5780	CTGATATGGGTGGAGAACAG	359	H. sapiens	714
216887	334	6353	TCTGGGACAGGTATGAGCTC	360	H. sapiens	715
216888	334	6534	TGATAGCAGTGGCCCTTGAA	361	H. sapiens	716
216889	334	6641	GGATTGGCGTGAAATACTGG	362	H. sapiens	717
216890	334	6661	TGCCCGAGGTTCCTCCTGCC	363	H. sapiens	718
216894	334	7059	GCACTAGCAAGACCACACTC	367	H. sapiens	719
216895	334	7066	CAAGACCACACTCTGCATAG	368	H. sapiens	720
216897	334	7209	TCCTCCATAGGATACCGTGT	370	H. sapiens	721
216899	334	7702	GGATGTAGGGCAGCAAAACC	372	H. sapiens	722
216900	334	7736	TCTGCACAAGGACTCCTTGT	373	H. sapiens	723
216901	334	8006	CAGCCTGTCTCAGTGAACAT	374	H. sapiens	724
216903	334	8239	CAGGATGCTTCCAGTCTAAT	376	H. sapiens	725
216904	334	8738	AAATGCTCGTCTCCAATCTC	377	H. sapiens	726
216906	334	9208	AACTTGTGTATCCAAATCCA	379	H. sapiens	727
216908	334	9545	TGACATGGTGTGCTTCCTTG	381	H. sapiens	728
216910	334	9770	ACACTGGTGTTCTGGCTACC	383	H. sapiens	729
216911	334	9776	GTGTTCTGGCTACCTCTAGT	384	H. sapiens	730
216913	334	10341	TCCTGGCATAGGTCACAGTA	386	H. sapiens	731
216914	334	10467	ATGTCAACAGTAGCACCTCC	387	H. sapiens	732
216915	334	10522	TAGACTCAGACAAGTCTGGA	388	H. sapiens	733
216917	334	10587	CCTAAGTTGCTCATCTCTGG	390	H. sapiens	734
216922	334	11337	TGCGCTGAGTTCCATGAAAC	395	H. sapiens	735
216923	334	11457	CTATTGCAGGTGCTCTCCAG	396	H. sapiens	736

216926	334	12155	CAGAGGAACATCTTGCACCT	399	*H. sapiens*	737
216928	334	12221	CTCTGCTCCTTACTCTTGTG	401	*H. sapiens*	738
216929	334	12987	GTAATTTCTCACCATCCATC	402	*H. sapiens*	739
216930	334	13025	TTCTGAGTCTCAATTGTCTA	403	*H. sapiens*	740
216931	334	13057	CTATGTCCTTGTGTGCACAT	404	*H. sapiens*	741
216932	334	13634	GCCTATTGCCATTTGTATGT	405	*H. sapiens*	742
216933	334	13673	CTATTCATGTCCTTTGCCTA	406	*H. sapiens*	743
216935	334	14567	GATTCTGCGGGTAATCTCAG	408	*H. sapiens*	744
216937	334	14680	TCAATGCAGGTCATTGGAAA	410	*H. sapiens*	745
216938	334	15444	CTACCAGATTGACCATCCCT	411	*H. sapiens*	746
216940	334	15757	TACTTGATAGTGCTCTAGGA	413	*H. sapiens*	747
216941	334	15926	TTGACTGCAGGACCAGGAGG	414	*H. sapiens*	748
216942	334	16245	AACAAACACTTGTGCAAATG	415	*H. sapiens*	749
216950	334	18519	AATGAGACCAAACTTCCACT	423	*H. sapiens*	750
216951	334	18532	TTCCACTTTGAAGCTAGCAA	424	*H. sapiens*	751
216952	334	18586	GATCTGGAGCTTATTCTTGA	425	*H. sapiens*	752
216953	334	18697	ACCTCATGTGACTTGTATGC	426	*H. sapiens*	753
216954	334	18969	TTCTTAAGAAACACCTTGTA	427	*H. sapiens*	754
216955	334	19250	TAGGCCCATCCTGGCTGCAT	428	*H. sapiens*	755
216956	334	19340	AAACTCTCAGGATATGGTAA	429	*H. sapiens*	756
216957	334	19802	ATACCTTCCTCTACCTTTGC	430	*H. sapiens*	757
216958	334	19813	TACCTTTGCTGAAGGTCCTT	431	*H. sapiens*	758
216961	334	20567	ATCTATCTAGTGAAATTTCT	434	*H. sapiens*	759
216962	334	20647	TCAGCTCATCAAAATATGCT	435	*H. sapiens*	760
216963	334	20660	ATATGCTAGTCCTTCCTTTC	436	*H. sapiens*	761
216965	334	21316	CAAAGGTCTGAGTTATCCAG	438	*H. sapiens*	762
216967	334	21422	TGACTTATAGATGCAGGCTG	440	*H. sapiens*	763
216968	334	21634	TCAGTGGAGGGTAATTCTTT	441	*H. sapiens*	764
216969	334	21664	TGCCTAGCCAGTTTGAAAGA	442	*H. sapiens*	765
216970	334	21700	CCTGCAGAATTTTGCCAGGC	443	*H. sapiens*	766
216972	334	22048	GTAGCTAGGTAGGTAAAGCA	445	*H. sapiens*	767
216973	334	22551	TTGAGTGAGACACACAAGGT	446	*H. sapiens*	768
216974	334	22694	GTGCTAGTCAGGGAATGCAT	447	*H. sapiens*	769
216976	334	22903	GGGGAGAGAGCATGCCCAGC	449	*H. sapiens*	770
216977	334	22912	GCATGCCCAGCTGCGAAAGC	450	*H. sapiens*	771
216978	334	23137	AGCCAGGTATAGAAAGGAGT	451	*H. sapiens*	772
216979	334	23170	AACTTTCTAAGAGGCAGAAT	452	*H. sapiens*	773
216981	334	23882	TCTTAGTCTGGTCATGAGTG	454	*H. sapiens*	774
216983	334	24184	AGTAGGAGATTTCATATGAA	456	*H. sapiens*	775
216985	334	24559	TCTTCACCAGCAACACATTA	458	*H. sapiens*	776
216987	334	24800	ATGGCCACCTAGCATGGCAC	460	*H. sapiens*	777
216989	334	24991	CATGTTTCTGAGCCTCCAGA	462	*H. sapiens*	778
216990	334	25067	TAGGTGGCTCCCTGTCTTCA	463	*H. sapiens*	779
216991	334	25152	TCCAAAGTCTTGGGAATCCT	464	*H. sapiens*	780
216995	334	26749	ACAAAGAAAGGGGGAGTTGG	468	*H. sapiens*	781
216996	334	26841	TCGTGTCTTCCTGGCCCAGA	469	*H. sapiens*	782
216997	334	27210	GCAGTGCCCAGCACACAATA	470	*H. sapiens*	783
216998	334	27815	ACTCGTCCAGGTGCGAAGCA	471	*H. sapiens*	784

216999	334	28026	GCCACCTAAGGTAAAGAAGG	472	*H. sapiens*	785
217000	334	28145	ATCAGAGTGGCAGAGAGAGC	473	*H. sapiens*	786
217002	334	28919	TTTACCATAGTTGTGACACA	475	*H. sapiens*	787
217006	334	29871	CATTTTGTAGGCAATGAGCT	479	*H. sapiens*	788
217007	334	30181	GCATTAGTAAACATGAGAAC	480	*H. sapiens*	789
217009	334	30931	TTCATTTCAGCGATGGCCGG	482	*H. sapiens*	790
217013	334	39562	GAAAATCTAGTGTCATTCAA	486	*H. sapiens*	791
217016	334	39789	TCCTATACAGTTTTGGGAAC	489	*H. sapiens*	792
217017	334	39904	AAGGACTTCAGTATGGAGCT	490	*H. sapiens*	793
217018	334	39916	ATGGAGCTTTTATTGAATTG	491	*H. sapiens*	794
217022	334	40412	CCATCAGCACTATTATTTAT	495	*H. sapiens*	795
217023	334	40483	ATAGGCAAGCTCAGCCATAG	496	*H. sapiens*	796
217025	334	40576	TGCTAGATGAGATACATCAA	498	*H. sapiens*	797
217026	334	40658	GAAGACCAAACATGGTTCTA	499	*H. sapiens*	798
217029	334	41130	CTCTGTTTAGTCCTCTCCAG	502	*H. sapiens*	799
217032	503	490	CATTGATAAAATGTTCTGGC	505	*H. sapiens*	800
217033	503	504	TCTGGCACAGCAAAACCTCT	506	*H. sapiens*	801
217034	503	506	TGGCACAGCAAAACCTCTAG	507	*H. sapiens*	802
217037	503	523	TAGAACACATAGTGTGATTT	510	*H. sapiens*	803
217039	503	526	AACACATAGTGTGATTTAAG	512	*H. sapiens*	804

As these "preferred target segments" have been found by experimentation to be open to, and accessible for, hybridization with the antisense compounds of the present invention, one of skill in the art will recognize or be able to ascertain, using no more than routine experimentation, further embodiments of the invention that encompass other compounds that specifically hybridize to these preferred target segments and consequently inhibit the expression of apolipoprotein B.

According to the present invention, antisense compounds include antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other short oligomeric compounds which hybridize to at least a portion of the target nucleic acid.

Example 37

Antisense inhibition of human apolipoprotein B expression -

dose response of oligonucleotides

In accordance with the present invention, 12 oligonucleotides described in Examples 29 and 31 were further investigated in a dose response study. The control oligonucleotides used in this study were ISIS 18076 (SEQ ID NO: 805) and ISIS 13650 (SEQ ID NO: 806).

All compounds in this study, including the controls, were chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotides. All cytidine residues are 5-methylcytidines.

In the dose-response experiment, with mRNA levels as the endpoint, HepG2 cells were treated with the antisense oligonucleotides or the control oligonucleotides at doses of 37, 75, 150, and 300 nM oligonucleotide. Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from two experiments with mRNA levels in the treatment groups being normalized to an untreated control group. The data are shown in Table 19.

Table 19

Inhibition of apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap - Dose Response

	Dose				
	37 nM	75 nM	150 nM	300 nM	
ISIS #	% inhibition				SEQ ID NO
271009	82	91	94	96	319

281625	62	76	84	94	224
301014	75	90	96	98	249
301027	80	90	95	96	262
301028	70	79	85	92	263
301029	54	67	79	85	264
301030	64	75	87	92	265
301031	61	82	92	96	266
301034	73	87	93	97	269
301036	67	83	92	95	271
301037	73	85	89	96	272
301045	77	86	94	98	280

Example 38

Antisense inhibition of human apolipoprotein B expression - dose response - Lower dose range

In accordance with the present invention, seven oligonucleotides described in Examples 29, 31, 35, and 36 were further investigated in a dose response study. The control oligonucleotides used in this study were ISIS 18076 (SEQ ID NO: 805), ISIS 13650 (SEQ ID NO: 806), and ISIS 129695 (SEQ ID NO: 807).

All compounds in this study, including the controls, were chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotides. All cytidine residues are 5-methylcytidines.

In the dose-response experiment, with mRNA levels as the endpoint, HepG2 cells were treated with the antisense oligonucleotides or the control oligonucleotides at doses of 12.5, 37, 75, 150, and 300 nM oligonucleotide. Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from two experiments

with mRNA levels in the treatment groups being normalized to an untreated control group. The data are shown in Table 20.

Table 20

Inhibition of apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap - Dose Response

	Dose					
	12.5 nM	**37 nM**	**75 nM**	**150 nM**	**300 nM**	
ISIS #	**% inhibition**					**SEQ ID #**
271009	67	86	92	94	95	319
281625	44	66	83	85	94	224
301012	63	79	90	92	95	247
308638	42	73	91	96	97	247
308642	59	84	91	97	98	319
308651	57	76	84	90	88	319
308658	29	61	73	78	90	224

Example 39

RNA Synthesis

In general, RNA synthesis chemistry is based on the selective incorporation of various protecting groups at strategic intermediary reactions. Although one of ordinary skill in the art will understand the use of protecting groups in organic synthesis, a useful class of protecting groups includes silyl ethers. In particular bulky silyl ethers are used to protect the 5′-hydroxyl in combination with an acid-labile orthoester protecting group on the 2′-hydroxyl. This set of protecting groups is then used with standard solid-phase synthesis technology. It is important to lastly remove the acid labile orthoester protecting group after all other synthetic steps. Moreover, the early use of the silyl protecting groups during synthesis ensures

facile removal when desired, without undesired deprotection of 2′ hydroxyl.

Following this procedure for the sequential protection of the 5′-hydroxyl in combination with protection of the 2′-hydroxyl by protecting groups that are differentially removed and are differentially chemically labile, RNA oligonucleotides were synthesized.

RNA oligonucleotides are synthesized in a stepwise fashion. Each nucleotide is added sequentially (3′- to 5′-direction) to a solid support-bound oligonucleotide. The first nucleoside at the 3′-end of the chain is covalently attached to a solid support. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are added, coupling the second base onto the 5′-end of the first nucleoside. The support is washed and any unreacted 5′-hydroxyl groups are capped with acetic anhydride to yield 5′-acetyl moieties. The linkage is then oxidized to the more stable and ultimately desired P(V) linkage. At the end of the nucleotide addition cycle, the 5′-silyl group is cleaved with fluoride. The cycle is repeated for each subsequent nucleotide.

Following synthesis, the methyl protecting groups on the phosphates are cleaved in 30 minutes utilizing 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (S_2Na_2) in DMF. The deprotection solution is washed from the solid support-bound oligonucleotide using water. The support is then treated with 40% methylamine in water for 10 minutes at 55 °C. This releases the RNA oligonucleotides into solution, deprotects the exocyclic amines, and modifies the 2′- groups. The oligonucleotides can be analyzed by anion exchange HPLC at this stage.

The 2′-orthoester groups are the last protecting groups to be removed. The ethylene glycol monoacetate orthoester protecting group developed by Dharmacon Research, Inc. (Lafayette, CO), is one example of a useful orthoester protecting group which, has the following important properties. It is stable to the conditions of nucleoside phosphoramidite synthesis and oligonucleotide synthesis. However, after oligonucleotide synthesis the oligonucleotide is treated with methylamine which not only cleaves the oligonucleotide from the solid support but also removes the acetyl groups from the orthoesters. The resulting 2-ethyl-hydroxyl substituents on the orthoester are less electron withdrawing than the acetylated precursor. As a result, the modified orthoester becomes more labile to acid-catalyzed hydrolysis. Specifically, the rate of cleavage is approximately 10 times faster after the acetyl groups are removed. Therefore, this orthoester possesses sufficient stability in order to be compatible with oligonucleotide synthesis and yet, when subsequently modified, permits deprotection to be carried out under relatively mild aqueous conditions compatible with the final RNA oligonucleotide product.

Additionally, methods of RNA synthesis are well known in the art (Scaringe, S. A. Ph.D. Thesis, University of Colorado, 1996; Scaringe, S. A., et al., *J. Am. Chem. Soc.*, **1998**, *120*, 11820-11821; Matteucci, M. D. and Caruthers, M. H. *J. Am. Chem. Soc.*, **1981**, *103*, 3185-3191; Beaucage, S. L. and Caruthers, M. H. *Tetrahedron Lett.*, **1981**, *22*, 1859-1862; Dahl, B. J., et al., *Acta Chem. Scand,*. **1990**, *44*, 639-641; Reddy, M. P., et al., *Tetrahedrom Lett.*, **1994**, *25*, 4311-4314; Wincott, F. et al., *Nucleic Acids Res.*, **1995**, *23*, 2677-2684; Griffin, B. E., et al., *Tetrahedron*, **1967**,

23, 2301-2313; Griffin, B. E., et al., *Tetrahedron*, **1967**, *23*, 2315-2331).

RNA antisense compounds (RNA oligonucleotides) of the present invention can be synthesized by the methods herein or purchased from Dharmacon Research, Inc (Lafayette, CO). Once synthesized, complementary RNA antisense compounds can then be stably annealed by methods known in the art to form double stranded (duplexed) antisense compounds. For example, duplexes can be formed by combining 30 µl of each of the complementary strands of RNA oligonucleotides (50 uM RNA oligonucleotide solution) and 15 µl of 5X annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) followed by heating for 1 minute at 90°C, then 1 hour at 37°C. The resulting duplexed antisense compounds can be used in kits, assays, screens, or other methods to investigate the role of a target nucleic acid.

Example 40

Design and screening of duplexed antisense compounds targeting apolipoprotein B

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements are designed to target apolipoprotein B. The nucleobase sequence of the antisense strand of the duplex comprises at least a portion of an oligonucleotide described herein. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For

example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini. The antisense and sense strands of the duplex comprise from about 17 to 25 nucleotides, or from about 19 to 23 nucleotides. Alternatively, the antisense and sense strands comprise 20, 21 or 22 nucleotides.

For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

```
  cgagaggcggacgggaccgTT    Antisense Strand
  |||||||||||||||||||
TTgctctccgcctgccctggc      Complement
```

In another embodiment, a duplex comprising an antisense strand having the same sequence CGAGAGGCGGACGGGACCG may be prepared with blunt ends (no single stranded overhang) as shown:

```
cgagaggcggacgggaccg    Antisense Strand
|||||||||||||||||||
gctctccgcctgccctggc    Complement
```

RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are stably annealed. The single strands are aliquoted and diluted to a concentration of 50 uM. Once diluted, 30 uL of each strand is combined with 15uL of a 5X solution of annealing buffer. The final concentration of said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2mM magnesium acetate. The final volume is 75 uL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the

dsRNA duplexes are used in experimentation. The final concentration of the dsRNA duplex is 20 uM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate apolipoprotein B expression.

When cells reached 80% confluency, they are treated with duplexed antisense compounds of the invention. For cells grown in 96-well plates, wells are washed once with 200 µL OPTI-MEM-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM-1 containing 12 µg/mL LIPOFECTIN (Gibco BRL) and the desired duplex antisense compound at a final concentration of 200 nM. After 5 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after treatment, at which time RNA is isolated and target reduction measured by RT-PCR.

Example 41

Design of phenotypic assays and *in vivo* studies for the use of apolipoprotein B inhibitors

Phenotypic assays

Once apolipoprotein B inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition. Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association of apolipoprotein B in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial

vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with apolipoprotein B inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest.

Analysis of the genotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the

efficacy or potency of the apolipoprotein B inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.

In vivo studies

The individual subjects of the *in vivo* studies described herein are warm-blooded vertebrate animals, which includes humans.

The clinical trial is subjected to rigorous controls to ensure that individuals are not unnecessarily put at risk and that they are fully informed about their role in the study.

To account for the psychological effects of receiving treatments, volunteers are randomly given placebo or apolipoprotein B inhibitor. Furthermore, to prevent the doctors from being biased in treatments, they are not informed as to whether the medication they are administering is a apolipoprotein B inhibitor or a placebo. Using this randomization approach, each volunteer has the same chance of being given either the new treatment or the placebo.

Volunteers receive either the apolipoprotein B inhibitor or placebo for eight week period with biological parameters associated with the indicated disease state or condition being measured at the beginning (baseline measurements before any treatment), end (after the final treatment), and at regular intervals during the study period. Such measurements include the levels of nucleic acid molecules encoding apolipoprotein B or apolipoprotein B protein levels in body fluids, tissues or organs compared to pre-treatment levels. Other measurements include, but

are not limited to, indices of the disease state or condition being treated, body weight, blood pressure, serum titers of pharmacologic indicators of disease or toxicity as well as ADME (absorption, distribution, metabolism and excretion) measurements.

Information recorded for each patient includes age (years), gender, height (cm), family history of disease state or condition (yes/no), motivation rating (some/moderate/great) and number and type of previous treatment regimens for the indicated disease or condition.

Volunteers taking part in this study are healthy adults (age 18 to 65 years) and roughly an equal number of males and females participate in the study. Volunteers with certain characteristics are equally distributed for placebo and apolipoprotein B inhibitor treatment. In general, the volunteers treated with placebo have little or no response to treatment, whereas the volunteers treated with the apolipoprotein B inhibitor show positive trends in their disease state or condition index at the conclusion of the study.

Example 42

Antisense inhibition of rabbit apolipoprotein B expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides was designed to target different regions of rabbit apolipoprotein B, using published sequences (GenBank accession number X07480.1, incorporated herein as SEQ ID NO: 808, GenBank accession number M17780.1, incorporated herein as SEQ ID NO: 809, and a sequence was derived using previously described primers (Tanaka, *Journ.*

Biol. Chem., **1993**, *268*, 12713-12718) representing an mRNA of the rabbit apolipoprotein B, incorporated herein as SEQ ID NO: 810). The oligonucleotides are shown in Table 21. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 21 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on rabbit apolipoprotein B mRNA levels in primary rabbit hepatocytes by quantitative real-time PCR as described in other examples herein. Primary rabbit hepatocytes were treated with 150 nM of the compounds in Table 21. For rabbit apolipoprotein B the PCR primers were:
forward primer: AAGCACCCCCAATGTCACC (SEQ ID NO: 811)
reverse primer: GGGATGGCAGAGCCAATGTA (SEQ ID NO: 812) and the PCR probe was: FAM- TCCTGGATTCAAGCTTCTATGTGCCTTCA -TAMRA (SEQ ID NO: 813) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 21

Inhibition of rabbit apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE

wings and a deoxy gap

ISIS #	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
233149	808	1	TGCTTGGAGAAGGTAAGATC	0	814
233150	810	1	GCGTTGTCTCCGATGTTCTG	20	815
233151	809	13	TAATCATTAACTTGCTGTGG	20	816
233152	808	22	TCAGCACGTAGCAATGCATT	0	817
233153	808	31	GCCTGATACTCAGCACGTAG	0	818
233154	809	31	CAATTGAATGTACTCAGATA	18	819
233155	808	51	ACCTCAGTGACTTGTAATCA	47	820
233156	809	51	CACTGGAAACTTGTCTCTCC	23	821
233157	809	71	AGTAGTTAGTTTCTCCTTGG	0	822
233159	808	121	TCAGTGCCCAAGATGTCAGC	0	823
233160	810	121	ATTGGAATAATGTATCCAGG	81	824
233161	809	130	TTGGCATTATCCAATGCAGT	28	825
233162	808	151	GTTGCCTTGTGAGCAGCAGT	0	826
233163	810	151	ATTGTGAGTGGAGATACTTC	80	827
233164	809	171	CATATGTCTGAAGTTGAGAC	8	828
233165	808	181	GTAGATACTCCATTTTGGCC	0	829
233166	810	181	GGATCACATGACTGAATGCT	82	830
233167	808	201	TCAAGCTGGTTGTTGCACTG	28	831
233168	808	211	GGACTGTACCTCAAGCTGGT	0	832
233169	808	231	GCTCATTCTCCAGCATCAGG	14	833
233170	809	251	TTGATCTATAATACTAGCTA	23	834
233172	810	282	ATGGAAGACTGGCAGCTCTA	86	835
233173	808	301	TTGTGTTCCTTGAAGCGGCC	3	836
233174	809	301	TGTGCACGGATATGATAACG	21	837
233175	810	306	GACCTTGAGTAGATTCCTGG	90	838
233176	810	321	GAAATCTGGAAGAGAGACCT	62	839
233177	808	331	GTAGCTTTCCCATCTAGGCT	0	840
233178	808	346	GATAACTCTGTGAGGGTAGC	0	841
233179	810	371	ATGTTGCCCATGGCTGGAAT	65	842
233180	809	381	AAGATGCAGTACTACTTCCA	13	843
233181	808	382	GCACCCAGAATCATGGCCTG	0	844
233182	809	411	CTTGATACTTGGTATCCACA	59	845
233183	810	411	CAGTGTAATGATCGTTGATT	88	846
233184	810	431	TAAAGTCCAGCATTGGTATT	69	847
233185	810	451	CAACAATGTCTGATTGGTTA	73	848
233186	810	473	GAAGAGGAAGAAAGGATATG	60	849
233187	810	481	TGACAGATGAAGAGGAAGAA	66	850
233188	810	500	TTGTACTGTAGTGCATCAAT	74	851
233189	809	511	GCCTCAATCTGTTGTTTCAG	46	852
233190	810	520	ACTTGAGCGTGCCCTCTAAT	69	853
233191	809	561	GAAATGGAATTGTAGTTCTC	31	854

Example 43

Antisense inhibition of rabbit apolipoprotein B expression

by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap-Dose Response Study

In accordance with the present invention, a subset of the antisense oligonuclotides in Example 42 was further investigated in dose-response studies. Treatment doses were 10, 50, 150 and 300 nM. ISIS 233160 (SEQ ID NO: 824), ISIS 233166 (SEQ ID NO: 830), ISIS 233172 (SEQ ID NO: 835), ISIS 233175 (SEQ ID NO: 838), and ISIS 233183 (SEQ ID NO: 846) were analyzed for their effect on rabbit apolipoprotein B mRNA levels in primary rabbit hepatocytes by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments and are shown in Table 22.

Table 22

Inhibition of rabbit apolipoprotein B mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

	Percent Inhibition			
ISIS #	**300 nM**	**150 nM**	**50 nM**	**10 nM**
233160	80	74	67	33
233166	73	79	81	66
233172	84	81	76	60
233175	93	90	85	67
233183	80	81	71	30

Example 44

Effects of antisense inhibition of apolipoprotein B in LDLr-/- mice - Dose Response

LDL receptor-deficient mice (LDLr(-/-)mice), a strain that cannot edit the apolipoprotein B mRNA and therefore synthesize exclusively apolipoprotein B-100, have markedly elevated LDL cholesterol and apolipoprotein B-100 levels and develop extensive atherosclerosis.

LDLr(-/-) mice, purchased from Taconic (Germantown, NY) were used to evaluate antisense oligonucleotides for their potential to lower apolipoprotein B mRNA or protein levels, as well as phenotypic endpoints associated with apolipoprotein B. LDLr(-/-) mice were separated into groups of males and females. LDLr(-/-) mice were dosed intraperitoneally twice a week for six weeks with either 10, 25, or 50 mg/kg of ISIS 147764 (SEQ ID NO: 109) or ISIS 270906 (SEQ ID NO: 856) which is a 4 base mismatch of ISIS 147764, or with saline, or 20 mg/kg of Atorvastatin. At study termination animals were sacrificed and evaluated for several phenotypic markers.

ISIS 147764 was able to lower cholesterol, triglycerides, and mRNA levels in a dose-dependent manner in both male and female mice while the 4-base mismatch ISIS 270906 was not able to do this. The results of the study are summarized in Table 23.

Table 23

Effects of ISIS 147764 treatment in male and female LDLr-/- mice on apolipoprotein B mRNA, liver enzyme, cholesterol, and triglyceride levels.

	Dose	Liver Enzymes IU/L		Lipoproteins mg/dL				mRNA %
ISIS No.	mg/kg	AST	ALT	CHOL	HDL	LDL	TRIG	control
Males								
Saline		68.4	26.6	279.2	125.4	134.7	170.6	100.0
147764	10	57.6	29.8	314.2	150.0	134.7	198.6	61.7
	25	112.6	78.8	185.0	110.6	66.2	104.2	30.7
	50	163.6	156.8	165.6	107.8	51.2	113.4	16.6
270906	50	167.4	348.0	941.0	244.2	541.9	844.8	N.D.
Atorvastatin	20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	110.9
Females								
Saline		65.0	23.4	265.8	105.8	154.9	121.4	100.0
147764	10	82.0	27.2	269.6	121.0	127.8	140.8	64.2
	25	61.4	32.2	175.8	99.5	68.9	100.4	41.3
	50	134.6	120.4	138.2	92.2	45.9	98.0	18.5
270906	50	96.0	88.6	564.6	200.0	310.0	240.4	N.D.

Atorvastatin	20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	109.0

Example 45

Effects of antisense inhibition of apolipoprotein B in Cynomolgus monkeys

Cynomolgus monkeys fed an atherogenic diet develop atherosclerosis with many similarities to atherosclerosis of human beings. Female Cynomolgus macaques share several similarities in lipoproteins and the cardiovascular system with humans. In addition to these characteristics, there are similarities in reproductive biology. The Cynomolgus female has a 28-day menstrual cycle like that of women. Plasma hormone concentrations have been measured throughout the Cynomolgus menstrual cycle, and the duration of the follicular and luteal phases, as well as plasma estradiol and progesterone concentrations across the cycle, are also remarkably similar to those in women.

Cynomolgus monkeys (male or female) can be used to evaluate antisense oligonucleotides for their potential to lower apolipoprotein B mRNA or protein levels, as well as phenotypic endpoints associated with apolipoprotein B including, but not limited to cardiovascular indicators, atherosclerosis, lipid diseases, obesity, and plaque formation. One study could include normal and induced hypercholesterolemic monkeys fed diets that are normal or high in lipid and cholesterol. Cynomolgus monkeys can be dosed in a variety of regimens, one being subcutaneously with 10-20 mg/kg of the oligomeric compound for 1-2 months. Parameters that may observed during the test period could include: total plasma cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, arterial wall cholesterol content, and coronary intimal thickening.

Example 46

Sequencing of Cynomolgus monkey (Macaca fascicularis) apolipoprotein B preferred target segment

In accordance with the present invention, a portion of the cynomolgus monkey apolipoprotein B mRNA not available in the art, was amplified. Positions 2920 to 3420 of the human apolipoprotein B mRNA sequence (GenBank accession number NM_000384.1, incorporated herein as SEQ ID NO: 3) contain the preferred target segment to which ISIS 301012 hybridizes and the corresponding segment of cynomolgus monkey apolipoprotein B mRNA was amplified and sequenced. The site to which ISIS 301012 hybridizes in the human apolipoprotein B was amplified by placing primers at 5' position 2920 and 3' position 3420. The cynomolgus monkey hepatocytes were purchased from In Vitro Technologies (Gaithersburg, MD). The 500 bp fragments were produced using human and cynomolgus monkey 1° hepatocyte cDNA and were produced by reverse transcription of purified total RNA followed by 40 rounds of PCR amplification. Following gel purification of the human and cynomolgus amplicons, the forward and reverse sequencing reactions of each product were performed by Retrogen (Invitrogen kit was used to create the single-stranded cDNA and provided reagents for Amplitaq PCR reaction). This cynomolgus monkey sequence is incorporated herein as SEQ ID NO: 855 and is 96% identical to positions 2920 to 3420 of the human apolipoprotein B mRNA.

Example 47

Effects of antisense inhibition of human apolipoprotein B gene (ISIS 281625 and 301012) in C57BL/6NTac-TgN(APOB100) transgenic mice

C57BL/6NTac-TgN(APOB100) transgenic mice have the human apolipoprotein B gene "knocked-in". These mice express high levels of human apolipoprotein B100 resulting in mice with elevated serum levels of LDL cholesterol. These mice are useful in identifying and evaluating compounds to reduce elevated levels of LDL cholesterol and the risk of atherosclerosis. When fed a high fat cholesterol diet, these mice develop significant foam cell accumulation underlying the endothelium and within the media, and have significantly more complex atherosclerotic lesions than control animals.

C57BL/6NTac-TgN(APOB100) mice were divided into two groups - one group receiving oligonucleotide treatment and control animals receiving saline treatment. After overnight fasting, mice were dosed intraperitoneally twice a week with saline or 25 mg/kg ISIS 281625 (SEQ ID No: 224) or ISIS 301012 (SEQ ID No: 247) for eight weeks. At study termination and forty eight hours after the final injections, animals were sacrificed and evaluated for target mRNA levels in liver, cholesterol and triglyceride levels, and liver enzyme levels. In addition, the endogenous mouse apolipoprotein B levels in liver were measured to evaluate any effects of these antisense oligonucletides targeted to the human apolipoprotein B.

Upon treatment with either ISIS 281625 or ISIS 301012, the AST and ALT levels were increased, yet did not exceed normal levels (~300 IU/L). Cholesterol levels were slightly increased relative to saline treatment, while triglyceride levels were slightly decreased. Treatment with either of these oligonucleotides targeted to the human apolipoprotein B which is expressed in these mice markedly decreased the mRNA levels of the human apolipoprotein,

while the levels of the endogenous mouse apolipoprotein B were unaffected, indicating that these oligonucleotides exhibit specificity for the human apolipoprotein B. The results of the comparative studies are shown in Table 24.

Table 24

Effects of ISIS 281625 and 301012 treatment in mice on apolipoprotein B mRNA, liver enzyme, cholesterol, and triglyceride levels.

		ISIS No.	
	SALINE	**281625**	**301012**
Liver Enzymes IU/L			
AST	70.3	265.8	208.4
ALT	32.8	363.8	137.4
Lipoproteins mg/dL			
CHOL	109.5	152.0	145.1
HDL	67.3	84.6	98.6
LDL	30.2	49.8	36.6
TRIG	194.5	171.1	157.8
mRNA % control			
human mRNA	100.0	45.2	23.7
mouse mRNA	100.0	111.0	94.6

Following 2 and 4 weeks of ISIS 301012 treatment, LDL-cholesterol levels were significantly reduced to 22 mg/dL and 17 mg/dL, respectively.

Apolipoprotein B protein levels in liver were also evaluated at the end of the 8 week treatment period. Liver protein was isolated and subjected to immunoblot analysis using antibodies specific for human or mouse apolipoprotein B protein (US Biologicals, Swampscott, MA and Santa Cruz Biotechnology, Inc., Santa Cruz, CA, respectively). Immunoblot analysis of liver protein samples reveals a reduction in the expression of both forms of human apolipoprotein B , apolipoprotein B-100 and apolipoprotein

B-48. Mouse apolipoprotein B levels in liver were not significantly changed, as judged by immunoblot analysis.

Serum samples were also collected at 2, 4, 6 and 8 weeks and were evaluated for human apolipoprotein B expression by using a human apolipoprotein B specific ELISA kit (ALerCHEK Inc., Portland, ME). Quantitation of serum human apolipoprotein B protein by ELISA revealed that treatment with ISIS 281625 reduced serum human apolipoprotein B protein by 31, 26, 11 and 26% at 2, 4, 6 and 8 weeks, respectively, relative to saline-treated animals. Treatment with ISIS 301012 reduced serum human apolipoprotein B protein by 70, 87, 81 and 41% at 2, 4, 6 and 8 weeks, respectively, relative to saline-treated control animals. Serum from transgenic mice was also subjected to immunoblot analysis using both human and mouse specific apolipoprotein B antibodies (US Biologicals, Swampscott, MA and Santa Cruz Biotechnology, Inc., Santa Cruz, CA, respectively). Immunoblot analysis of serum samples taken from animals shows a similar pattern of human apolipoprotein B expression, with a significant reduction in serum apolipoprotein B protein after 2, 4 and 6 weeks of treatment and a slight reduction at 8 weeks. Mouse apolipoprotein B in serum was not significantly changed, as judged by immunoblot analysis.

Example 48

Effects of antisense inhibition of apolipoprotein B (ISIS 233172, 233175, 281625, 301012, and 301027) in C57BL/6 mice

C57BL/6 mice, a strain reported to be susceptible to hyperlipidemia-induced atherosclerotic plaque formation were used in the following studies to evaluate the toxicity in mice of several antisense oligonucleotides targeted to human or rabbit apolipoprotein B.

C57BL/6 mice were divided into two groups - one group receiving oligonucleotide treatment and control animals

receiving saline treatment. After overnight fasting, mice were dosed intraperitoneally twice a week with saline or 25 mg/kg of one of several oligonucleotides for two weeks. The antisense oligonucleotides used in the present study were ISIS 233172 (SEQ ID NO: 835) and ISIS 233175 (SEQ ID NO: 838), both targeted to rabbit apolipoprotein B, and ISIS 281625 (SEQ ID NO: 224), ISIS 301012 (SEQ ID NO: 247), and ISIS 301027 (SEQ ID NO: 262), targeted to human apolipoprotein B. At study termination and forty eight hours after the final injections, animals were sacrificed and evaluated for liver enzyme levels, body weight, liver weight, and spleen weight.

The levels of liver enzymes in mice were decreased relative to saline treatment for three of the antisense oligonucleotide. However, the rabbit oligonucleotide ISIS 233175 and the human oligonucleotide ISIS 301027 both elicited drastically increased levels of these liver enzymes, indicating toxicity. For all of the oligonucleotides tested, the change in weight of body, liver, and spleen were minor. The results of the comparative studies are shown in Table 25.

Table 25

Effects of antisense oligonucleotides targeted to human or rabbit apolipoprotein B on mouse apolipoprotein B mRNA, liver enzyme, cholesterol, and triglyceride levels.

			ISIS No.				
		SALINE	**233172**	**233175**	**281625**	**301012**	**301027**
Liver Enzymes							
AST	IU/L	104.5	94.3	346.7	89.5	50.6	455.3
ALT	IU/L	39.5	43.3	230.2	36.2	21.2	221.3
Weight							
BODY		21.2	21.3	21.5	20.9	21.3	21.2

LIVER	1.1	1.3	1.4	1.2	1.1	1.3
SPLEEN	0.1	0.1	0.1	0.1	0.1	0.1

Example 49

Time course evaluation of oligonucleotide at two different doses

C57BL/6 mice, a strain reported to be susceptible to hyperlipidemia-induced atherosclerotic plaque formation were used in the following studies to evaluate the toxicity in mice of several antisense oligonucleotides targeted to human apolipoprotein B.

Female C57BL/6 mice were divided into two groups - one group receiving oligonucleotide treatment and control animals receiving saline treatment. After overnight fasting, mice were dosed intraperitoneally twice a week with saline or 25 mg/kg or 50 mg/kg of ISIS 281625 (SEQ ID NO: 224), ISIS 301012 (SEQ ID NO: 247), or ISIS 301027 (SEQ ID NO: 262). After 2 weeks, a blood sample was taken from the tail of the mice and evaluated for liver enzyme. After 4 weeks, and study termination, animals were sacrificed and evaluated for liver enzyme levels.

For ISIS 281625 and ISIS 301012, AST and ALT levels remained close to those of saline at either dose after 2 weeks. After 4 weeks, AST and ALT levels showed a moderate increase over saline treated animals for the lower dose, but a large increase at the higher dose. ISIS 301027, administered at either dose, showed a small increase in AST and ALT levels after 2 weeks and a huge increase in AST and ALT levels after 4 weeks. The results of the studies are summarized in Table 26.

Table 26

AST and ALT levels in mice treated with ISIS 281625, 301012, or 301027 after 2 and 4 weeks

		AST (IU/L)		ALT (IU/L)	
		2 weeks	4 weeks	2 weeks	4 weeks
SALINE		49.6	63.2	22.4	25.2
ISIS No.	Dose (mg/kg)				
281625	25	40.8	75	21.2	31.8
	50	44.4	152.4	30.8	210.4
301012	25	37.2	89.8	22.4	24.8
	50	38.4	107.4	23.2	29.2
301027	25	55.4	537.6	27.2	311.2
	50	64	1884	34.8	1194

Example 50

Effects of antisense inhibition of apolipoprotein B (ISIS 147483 and 147764) in ob/ob mice

Leptin is a hormone produced by fat that regulates appetite. Deficiencies in this hormone in both humans and non-human animals leads to obesity. ob/ob mice have a mutation in the leptin gene which results in obesity and hyperglycemia. As such, these mice are a useful model for the investigation of obesity and diabetes and treatments designed to treat these conditions.

Ob/ob mice receiving a high fat, high cholesterol diet (60% kcal fat supplemented with 0.15% cholesterol) were treated with one of several oligonucleotides to evaluate their effect on apolipoprotein B-related phenotypic endpoints in ob/ob mice. After overnight fasting, mice from each group were dosed intraperitoneally twice a week with 50 mg/kg of ISIS 147483 (SEQ ID NO: 79), or 147764 (SEQ ID NO: 109), or the controls ISIS 116847 (SEQ ID NO: 857), or 141923 (SEQ ID NO: 858), or saline for six weeks. At study termination and forty eight hours after the final injections, animals were sacrificed and evaluated for

target mRNA levels in liver, cholesterol and triglyceride levels, liver enzyme levels, serum glucose levels, and PTEN levels.

ISIS 147483 and 147764 were both able to lower apolipoprotein B mRNA levels, as well as glucose, cholesterol, and triglyceride levels. The results of the comparative studies are shown in Table 27.

Table 27

Effects of ISIS 147483 and 147764 treatment in ob/ob mice on apolipoprotein B mRNA, cholesterol, lipid, triglyceride, liver enzyme, glucose, and PTEN levels.

			ISIS No.			
		SALINE	**116847**	**141923**	**147483**	**147764**
Glucose mg/dL		269.6	135.5	328.5	213.2	209.2
Liver Enzymes						
IU/L	AST	422.3	343.2	329.3	790.2	406.5
	ALT	884.3	607.5	701.7	941.7	835.0
Lipoproteins						
mg/dL	CHOL	431.9	287.5	646.3	250.0	286.3
	TRIG	128.6	196.5	196.5	99.8	101.2
mRNA	% control					
	ApoB	100.0	77.0	100.0	25.2	43.1
	PTEN	100.0	20.0	113.6	143.2	115.3

Example 51

Antisense inhibition of apolipoprotein B in high fat fed mice: time-dependent effects

In a further embodiment of the invention, the inhibition of apolipoprotein B mRNA in mice was compared to liver oligonucleotide concentration, total cholesterol, LDL-cholesterol and HDL-cholesterol. Male C57Bl/6 mice receiving a high fat diet (60% fat) were evaluated over the course of 6 weeks for the effects of treatment with twice weekly intraperitoneal injections of 50 mg/kg ISIS 147764

(SEQ ID NO: 109) or 50 mg/kg of the control oligonucleotide ISIS 141923 (SEQ ID NO: 858). Control animals received saline treatment. Animals were sacrificed after 2 days, 1, 2, 4 and 6 weeks of treatment. Each treatment group at each time point consisted of 8 mice.

Target expression in liver was measured by real-time PCR as described by other examples herein and is expressed as percent inhibition relative to saline treated mice. Total, LDL- and HDL-cholesterol levels were measured by routine clinical analysis using an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY) and are presented in mg/dL. Results from saline-treated animals are shown for comparison.. Intact oligonucleotide in liver tissue was measured by capillary gel electrophoresis and is presented as micrograms of oligonucleotide per gram of tissue. All results are the average of 8 animals and are shown in Table 28.

Table 28

Correlation between liver drug concentration, apolipoprotein B mRNA expression and serum lipids during ISIS 147764 treatment

		Treatment period				
	ISIS #	**2 days**	**1 week**	**2 weeks**	**4 weeks**	**6 weeks**
% Inhibition apolipoprotein B mRNA	**141923**	9	4	7	0	0
	147764	50	57	73	82	88
Intact oligonucleotide ug/g	**141923**	58	61	152	261	631
	147764	85	121	194	340	586
Total cholesterol mg/dL	**saline**	105	152	144	180	191
	141923	99	146	152	169	225
	147764	101	128	121	75	73
LDL-cholesterol	**saline**	8	32	28	50	46

mg/dL	141923	8	27	27	38	56
	147764	7	19	14	7	7
HDL-cholesterol mg/dL	saline	74	117	114	127	141
	141923	70	116	122	128	166
	147764	76	107	105	66	64

These results illustrate that inhibition of apolipoprotein B mRNA by ISIS 147764 occurred within 2 days of treatment, increased with successive treatments and persisted for 6 weeks of treatment. Quantitation of liver oligonucleotide levels reveals a strong correlation between the extent of target inhibition and liver drug concentration. Furthermore, at 1, 2, 3 and 4 weeks of treatment, a inverse correlation between inhibition of target mRNA and cholesterol levels (total, HDL and LDL) is observed, with cholesterol levels lowering as percent inhibition of apolipoprotein B mRNA becomes greater. Serum samples were subjected to immunoblot analysis using an antibody to detect mouse apolipoprotein B protein (Gladstone Institute, San Francisco, CA). The expression of protein follows the same pattern as that of the mRNA, with apolipoprotein B protein in serum markedly reduced within 48 hours and lowered throughout the 6 week treatment period.

The oligonucleotide treatments described in this example were duplicated to investigate the extent to which effects of ISIS 147764 persist following cessation of treatment. Mice were treated as described, and sacrificed 1, 2, 4, 6 and 8 weeks following the cessation of oligonucleotide treatment. The same parameters were analyzed and the results are shown in Table 29.

Table 29

Correlation between liver drug concentration, apolipoprotein B mRNA expression, and serum lipids after cessation of dosing

	ISIS #	Treatment period				
		1 week	2 weeks	4 weeks	6 weeks	8 weeks
% Inhibition apolipoprotein B mRNA	141923	15	2	7	11	7
	147764	82	78	49	37	19
Intact oligonucleotide ug/g	141923	297	250	207	212	128
	147764	215	168	124	70	43
Total cholesterol mg/dL	saline	114	144	195	221	160
	141923	158	139	185	186	151
	147764	69	67	111	138	135
LDL-cholesterol mg/dL	saline	21	24	34	37	22
	141923	24	24	32	32	24
	147764	14	14	18	24	21
HDL-cholesterol mg/dL	saline	86	109	134	158	117
	141923	121	105	135	136	108
	147764	51	49	79	100	94

These data demonstrate that after termination of oligonucleotide treatment, the effects of ISIS 147764, including apolipoprotein B mRNA inhibition, and cholesterol lowering, persist for up to 8 weeks. Immunoblot analysis demonstrates that apolipoprotein B protein levels follow a pattern similar that observed for mRNA expression levels.

Example 52

Effects of antisense inhibition of human apolipoprotein B gene by 301012 in C57BL/6NTac-TgN(APOB100) transgenic mice: dosing study

C57BL/6NTac-TgN(APOB100) transgenic mice have the human apolipoprotein B gene "knocked-in". These mice express high levels of human apolipoprotein B resulting in mice with elevated serum levels of LDL cholesterol. These mice are useful in identifying and evaluating compounds to

reduce elevated levels of LDL cholesterol and the risk of atherosclerosis. When fed a high fat cholesterol diet, these mice develop significant foam cell accumulation underlying the endothelium and within the media, and have significantly more complex atherosclerotic plaque lesions than control animals.

A long-term study of inhibition of human apolipoprotein B by ISIS 301012 in C57BL/6NTac-TgN(APOB100) mice (Taconic, Germantown, NY) was conducted for a 3 month period. Mice were dosed intraperitoneally twice a week with 10 or 25 mg/kg ISIS 301012 (SEQ ID No: 247) for 12 weeks. Saline-injected animals served as controls. Each treatment group comprised 4 animals.

After 2, 4, 6, 8 and 12 weeks of treatment, serum samples were collected for the purpose of measuring human apolipoprotein B protein. Serum protein was quantitated using an ELISA kit specific for human apolipoprotein B (ALerCHEK Inc., Portland, ME). The data are shown in Table 30 and each result represents the average of 4 animals. Data are normalized to saline-treated control animals.

Table 30

Reduction of human apolipoprotein B protein in transgenic mouse serum following ISIS 301012 treatment

	% Reduction in human apolipoprotein B protein in serum				
Dose of oligonucleotide mg/kg	2 weeks	4 weeks	6 weeks	8 weeks	12 weeks
10	76	78	73	42	85
25	80	87	86	47	79

These data illustrate that following 2, 4, 6 or 12 weeks of treatment with ISIS 301012, the level of human

apolipoprotein B protein in serum from transgenic mice is lowered by approximately 80%, demonstrating that in addition to inhibiting mRNA expression, ISIS 301012 effectively inhibits human apolipoprotein B protein expression in mice carrying the human apolipoprotein B transgene. Apolipoprotein B protein in serum was also assessed by immunoblot analysis using an antibody directed to human apolipoprotein B protein (US Biologicals, Swampscott, MA). This analysis shows that the levels human apolipoprotein B protein, both the apolipoprotein B-100 and apolipoprotein B-48 forms, are lowered at 2, 4, 6 and 12 weeks of treatment. Immunoblot analysis using a mouse apolipoprotein B specific antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) reveals no significant change in the expression of the mouse protein in serum.

At the beginning of the treatment (start) and after 2, 4, 6 and 8 weeks of treatment, serum samples were collected and total, LDL- and HDL-cholesterol levels were measured by routine clinical analysis using an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY), and these data are presented in Table 31. Results are presented as mg/dL in serum and represent the average of 4 animals. Results from the saline control animals are also shown.

Table 31

Effects of ISIS 301012 on serum lipids in human apolipoprotein B transgenic mice

		Treatment period				
	Treatment	**Start**	**2 weeks**	**4 weeks**	**6 weeks**	**8 weeks**
Total cholesterol mg/dL	**Saline**	120	110	129	121	126
	10	115	97	111	120	122
	25	107	101	107	124	147
HDL-cholesterol mg/dL	**Saline**	67	61	69	62	64
	10	70	69	78	72	79

	25	64	73	76	80	91
LDL-cholesterol mg/dL	Saline	39	41	50	45	47
	10	35	20	23	37	33
	25	33	19	19	37	44

These data demonstrate that LDL-cholesterol is lowered by treatment with 10 or 25 mg/kg of ISIS 147764 during the first 4 weeks of treatment.

The study was terminated forty eight hours after the final injections in the eighth week of treatment, when animals were sacrificed and evaluated for target mRNA levels in liver, apolipoprotein B protein levels in liver and serum cholesterol and liver enzyme levels. In addition, the expression of endogenous mouse apolipoprotein B levels in liver was measured to evaluate any effects of ISIS 301012 on mouse apolipoprotein B mRNA expression.

Human and mouse apolipoprotein B mRNA levels in livers of animals treated for 12 weeks were measured by real-time PCR as described herein. Each result represents the average of data from 4 animals. The data were normalized to saline controls and are shown in Table 32.

Table 32

Effects of ISIS 301012 on human and mouse apolipoprotein B mRNA levels in transgenic mice

	% Inhibition	
	Dose of ISIS 301012	
mRNA species measured	**10 mg/kg**	**25 mg/kg**
human apolipoprotein B	65	75
mouse apolipoprotein B	6	6

These data demonstrate that following 12 weeks of treatment with ISIS 301012, human apolipoprotein B mRNA is reduced by as much as 75% in the livers of transgenic mice,

whereas mouse liver apolipoprotein B mRNA was unaffected. Furthermore, ELISA analysis of apolipoprotein B protein in livers of transgenic mice reveals an 80% and 82% reduction in the human protein following 10 and 20 mg/kg ISIS 301012, respectively. Immunoblot analysis using an antibody directed to human apolipoprotein B also demonstrates a reduction in the expression of human apolipoprotein B, both the apolipoprotein B-100 and apolipoprotein B-48 forms, in the livers of transgenic mice. Immunoblot analysis using an antibody directed to mouse apolipoprotein B protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) reveals that expression of the mouse protein in liver does not change significantly.

ALT and AST levels in serum were also measured using the Olympus Clinical Analyzer (Olympus America Inc., Melville, NY) and showed that following treatment with ISIS 301012, the AST and ALT levels were increased, yet did not exceed normal levels (~300 IU/L), indicating a lack of toxicity due to ISIS 301012 treatment.

Example 53

Assessment of in vitro immunostimulatory effects of ISIS 301012

Immunostimulatory activity is defined by the production of cytokines upon exposure to a proinflammatory agent. In a further embodiment of the invention, ISIS 301012 was tested for immunostimulatory, or proinflammatory, activity. These studies were performed by MDS Pharma Services (Saint Germain sur l'Arbresle, France). Whole blood was collected from naive B6C3F1 mice, which had not been knowingly exposed to viral, chemical or radiation treatment. Cultured blood cells were exposed to 0.5, 5 or

50 µM of ISIS 301012 for a period of 14 to 16 hours. Antisense oligonucleotides known to possess proinflammatory activity served as positive controls. Each treatment was performed in triplicate. At the end of the treatment period, supernatants were collected and cytokine analysis was performed using a flow cytometry method with the mouse Inflammation CBA kit (Becton Dickinson, Franklin Lakes, NJ). The results revealed that ISIS 301012 does not stimulate the release of any of the tested cytokines, which were interleukin-12p70 (IL-12p70), tumor necrosis factor-alpha (TNF-alpha), interferon-gamma (IFN-gamma), interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1) and interleukin-10 (IL-10). Thus, ISIS 301012 does not possess immunostimulatory activity, as determined by the in vitro immunostimulatory assay.

Example 54

Comparative genomic analysis of apolipoprotein B

In accordance with the present invention, a comparative genomic analysis of apolipoprotein B sequences from human, mouse and monkey was performed and illustrated that apolipoprotein B sequences are conserved across species. The organization of human and mouse apolipoprotein B genes is also highly conserved. The human and mouse genes are comprised of 29 and 26 exons, respectively. The mouse mRNA is approximately 81% homologous to the human sequence. The complete sequence and gene structure of the apolipoprotein B gene in non-human primates have not been identified. However, as illustrated in Example 46, a 500 base pair fragment which contains the ISIS 301012 target sequence exhibits approximately 96% identity to the human sequence.

The binding site for ISIS 301012 lies within the coding region, within exon 22 of the human apolipoprotein B mRNA. When the ISIS 301012 binding sites from human, mouse and monkey were compared, significant sequence diversity was observed. Although the overall sequence conservation between human and monkey over a 500 nucleotide region was approximately 96%, the ISIS 301012 binding site of the monkey sequence contains 2 mismatches relative to the human sequence. Likewise, though the mouse apolipoprotein B mRNA sequence is approximately 81% homologous to human, within the ISIS 301012 binding site, 5 nucleotides are divergent. The sequence comparisons for the ISIS 301012 binding site for human, mouse and monkey apolipoprotein B sequences are shown in Table 33. Mismatched nucleotides relative to the ISIS 301012 target sequence are underlined.

Table 33
Comparison of ISIS 301012 binding site among human, monkey and mouse apolipoprotein B sequences

Species	# Mismatches	ISIS 301012 target sequence
Human	0	aggtgcgaagcagactgagg
Monkey	2	aggtgtaaagcagactgagg
Mouse	5	aggagtgcagcagtctgaag

The target sequence to which the mouse antisense oligonucleotide ISIS 147764 hybridizes lies within exon 24 of the mouse apolipoprotein B gene. The sequence comparisons for the ISIS 147764 binding site in mouse and human apolipoprotein B sequences are shown in Table 34. Mismatched nucleotides relative to the ISIS 147764 target sequence are underlined.

Table 34

Comparison of ISIS 147764 binding site between mouse and human apolipoprotein B sequences

Species	# Mismatches	ISIS 147764 binding site
Human	5	gcattgacatcttcagggac
Mouse	0	gcatggacttcttctggaaa

Example 55

BLAST analysis of ISIS 301012

In accordance with the present invention, the number of regions in the human genome to which ISIS 301012 will hybridize with perfect complementarity was determined. Percent complementarity of an antisense compound with a region of a target nucleic acid was determined using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., *J. Mol. Biol.*, **1990**, 215, 403-410; Zhang and Madden, *Genome Res.*, **1997**, 7, 649-656). This analysis assessed sequence complementarity in genomic or pre-mRNA regions and in coding sequences.

In genomic regions, ISIS 301012 shows perfect sequence complementarity to the apolipoprotein B gene only. No target sequences with one mismatch relative to ISIS 301012 were found. Two mismatches are found between the ISIS 301012 target sequence and the heparanase gene, and 3 mismatches are found between the ISIS 301012 target sequence and 28 unique genomic sites.

In RNA sequences, perfect sequence complementarity is found between ISIS 301012 and the apolipoprotein B mRNA and

three expressed sequence tags that bear moderate similarity to a human apolipoprotein B precursor. A single mismatch is found between ISIS 301012 and an expressed sequence tag similar to the smooth muscle form of myosin light chain.

Example 56

Antisense inhibition of apolipoprotein B in primary human hepatocytes: dose response studies

In accordance with the present invention, antisense oligonucleotides targeted to human apolipoprotein B were tested in dose response studies in primary human hepatocytes. Pre-plated primary human hepatocytes were purchased from InVitro Technologies (Baltimore, MD). Cells were cultured in high-glucose DMEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA), 100 units/mL and 100 µg/mL streptomycin (Invitrogen Corporation, Carlsbad, CA).

Human primary hepatocytes were treated with ISIS 301012 (SEQ ID NO: 247) at 10, 50, 150 or 300 nM. Untreated cells and cells treated with the scrambled control oligonucleotide ISIS 113529 (CTCTTACTGTGCTGTGGACA, SEQ ID NO: 859) served as two groups of control cells. ISIS 113529 is a chimeric oligonucleotide ("gapmer") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidines are 5-methylcytidines.

Oligonucleotides were introduced into cells through LIPOFECTIN-mediated transfection as described by other examples herein. Cells were harvested both 24 and 48 hours after treatment with oligonucleotide, and both RNA and protein were isolated. Additionally, the culture media from treated cells was collected for ELISA analysis of apolipoprotein B protein secretion.

Apolipoprotein B mRNA expression was determined by real-time PCR of RNA samples as described by other examples herein. Each result represents 6 experiments. The data are normalized to untreated control cells and are shown in Table 35.

Table 35

Inhibition of apolipoprotein B mRNA by antisense oligonucleotides in human primary hepatocytes

		% Inhibition of apolipoprotein B mRNA	
		ISIS #	
Dose of oligonucleotide	Treatment (hours)	301012	113529
10 nM	24	65	N.D.
	48	33	N.D.
50 nM	24	75	N.D.
	48	48	N.D.
150 nM	24	90	16
	48	78	5
300 nM	24	89	10
	48	72	18

These data demonstrate that ISIS 301012 inhibits apolipoprotein B expression in a dose-dependent manner in human primary hepatocytes.

Apolipoprotein B protein secreted from into the cultured cell media was measured in the samples treated with 50 and 150 nM of oligonucleotide, using a target protein specific ELISA kit (ALerCHEK Inc., Portland, ME).

Each result represents 3 experiments. The data are normalized to untreated control cells and are shown in Table 36.

Table 36

Inhibition of apolipoprotein B protein secretion from human primary hepatocytes by ISIS 301012

		% Change in apolipoprotein B protein secretion	
		ISIS #	
Dose	**Treatment (hours)**	**301012**	**113529**
150 nM	**24**	-57	+6
	48	-75	+4
300 nM	**24**	-41	-2
	48	-48	-5

Protein samples from 50, 150 and 300 nM doses after 24 hours and 150 and 300 nM doses after 48 hours were subjected to immunoblot analysis as described by other examples herein, using a human apolipoprotein B protein specific antibody purchased from US Biological (Swampscott, MA). Immunoblot analysis further demonstrates that apolipoprotein B protein in human hepatocytes is reduced in a dose-dependent manner following antisense oligonucleotide treatment with ISIS 301012.

An additional experiment was performed to test the effects of ISIS 271009 (SEQ ID NO: 319), ISIS 281625 (SEQ ID NO: 224) and ISIS 301027 (SEQ ID NO: 262) on human apolipoprotein B mRNA in human primary hepatocytes. Cells were cultured as described herein and treated with 5, 10, 50 or 150 nM of ISIS 271009, ISIS 281625 or ISIS 301027 for a period of 24 hours. The control oligonucleotides ISIS 13650 (SEQ ID NO: 806) and ISIS 113529 (SEQ ID NO: 859) were used at 50 or 150 nM. Human apolipoprotein B mRNA

expression was evaluated by real-time PCR as described by other examples herein. Apolipoprotein B protein secreted into the cultured cell media was measured in the samples treated with 50 and 150 nM of oligonucleotide, using a target protein specific ELISA kit (ALerCHEK Inc., Portland, ME).

The data, shown in Table 37, represent the average 2 experiments and are normalized to untreated control cells. Where present, a "+" indicates that gene expression was increased.

Table 37
Antisense inhibition of human apolipoprotein B mRNA by ISIS 271009, ISIS 281625 and ISIS 301027

	Oligonucleotide dose	ISIS 271009	ISIS 281625	ISIS 301027	ISIS 13650	ISIS 113529
% Inhibition of apolipoprotein B mRNA expression	**5 nM**	+4	8	11	N.D.	N.D.
	10 nM	5	22	37	N.D.	N.D.
	50 nM	52	49	50	38	0
	150 nM	81	52	70	26	14
% Inhibition of apolipoprotein B protein secretion	**50 nM**	17	18	21	N.D.	N.D.
	150 nM	32	18	32	+18	+1

These data demonstrate that ISIS 271009, ISIS 281625 and ISIS 301027 inhibit apolipoprotein B mRNA expression in a dose-dependent manner in human primary hepatocytes. ISIS 271009 and ISIS 301027 inhibit the secretion of apolipoprotein B protein from cells in a dose-dependent manner.

Example 57

Effects of apolipoproteinB-100 antisense oligonucleotides on apolipoprotein(a) expression

Lipoprotein(a) [Lp(a)] contains two disulfide-linked

distinct proteins, apolipoprotein(a) and apolipoprotein B (Rainwater and Kammerer, J. Exp. Zool., 1998, 282, 54-61). In accordance with the present invention, antisense oligonucleotides targeted to apolipoprotein B were tested for effects on the expression of the apolipoprotein(a) component of the lipoprotein(a) particle in primary human hepatocytes.

Primary human hepatocytes (InVitro Technologies, Baltimore, MD), cultured and transfected as described herein, were treated with 5, 10, 50 or 150 nM of ISIS 271009 (SEQ ID NO: 319), 281625 (SEQ ID NO: 224), 301012 (SEQ ID NO: 247) or 301027 (SEQ ID NO: 262). Cells were also treated with 50 or 150 nM of the control oligonucleotides ISIS 113529 (SEQ ID NO: 859) or ISIS 13650 (SEQ ID NO: 806). Untreated cells served as a control. Following 24 hours of oligonucleotide treatment, apolipoprotein(a) mRNA expression was measured by quantitative real-time PCR as described in other examples herein.

Probes and primers to human apolipoprotein(a) were designed to hybridize to a human apolipoprotein(a) sequence, using published sequence information (GenBank accession number NM_005577.1, incorporated herein as SEQ ID NO: 860). For human apolipoprotein(a) the PCR primers were:
forward primer: CAGCTCCTTATTGTTATACGAGGGA (SEQ ID NO: 861)
reverse primer: TGCGTCTGAGCATTGCGT (SEQ ID NO: 862) and the PCR probe was: FAM-CCCGGTGTCAGGTGGGAGTACTGC-TAMRA (SEQ ID NO: 863) where FAM is the fluorescent dye and TAMRA is the quencher dye.

Data are the average of three experiments and are expressed as percent inhibition relative to untreated

controls. The results are shown in Table 38. A "+" or "-" preceding the number indicates that apolipoprotein(a) expression was increased or decreased, respectively, following treatment with antisense oligonucleotides.

Table 38

Effects of apolipoprotein B antisense oligonucleotides on apolipoprotein(a) expression

	% Change in apolipoprotein(a) mRNA expression following antisense inhibition of apolipoprotein B					
Oligonucleotide Dose	**ISIS #**					
	271009	**281625**	**301012**	**301027**	**13650**	**113529**
5 nM	+70	-9	+34	-16	N.D.	N.D.
10 nM	+31	-23	+86	-45	N.D.	N.D.
50 nM	+25	-34	+30	-39	-68	+14
150 nM	-47	+32	+38	-43	-37	-9

These results illustrate that ISIS 301012 did not inhibit the expression of apolipoprotein(a) in human primary hepatocytes. ISIS 271009 inhibited apolipoprotein(a) expression at the highest dose. ISIS 281625 and ISIS 301027 decreased the levels of apolipoprotein(a) mRNA.

Example 58

Inhibition of lipoprotein(a) particle secretion with antisense oligonucleotides targeted to apolipoproteinB-100

In accordance with the present invention, the secretion of lipoprotein(a) particles, which are comprised of one apolipoprotein(a) molecule covalently linked to one apolipoprotein B molecule, was evaluated in primary human hepatocytes treated with antisense oligonucleotides targeted to the apolipoprotein B component of lipoprotein(a).

Primary human hepatocytes (InVitro Technologies, Baltimore, MD), cultured and transfected as described herein, were treated for 24 hours with 50 or 150 nM of ISIS 271009 (SEQ ID NO: 319), 281625 (SEQ ID NO: 224), 301012 (SEQ ID NO: 247) or 301027 (SEQ ID NO: 262). Cells were also treated with 150 nM of the control oligonucleotides ISIS 113529 (SEQ ID NO: 859) or ISIS 13650 (SEQ ID NO: 806). Untreated cells served as a control. Following 24 hours of oligonucleotide treatment, the amount of lipoprotein(a) in the culture medium collected from the treated cells was measured using a commercially available ELISA kit (ALerCHEK Inc., Portland, ME). The results are the average of three experiments and are expressed as percent change in lipoprotein(a) secretion relative to untreated controls. The data are shown in Table 39. A "+" or "-" preceding the number indicates that lipoprotein(a) particle secretion was increased or decreased, respectively, following treatment with antisense oligonucleotides targeted to apolipoprotein B.

Table 39

Inhibition of lipoprotein(a) particle secretion with antisense oligonucleotides targeted to apolipoprotein B

	% Change in lipoprotein(a) secretion					
Oligonucleotide Dose	**ISIS #**					
	271009	**281625**	**301012**	**301027**	**13650**	**113529**
50 nM	-25	-26	-27	-33	N.D.	N.D.
150 nM	-42	-24	-37	-44	+14	+14

These data demonstrate that antisense inhibition of apolipoprotein B, a component of the lipoprotein(a) particle, can reduce the secretion of lipoprotein(a) from human primary hepatocytes. In addition, this reduction in

lipoprotein(a) secretion is not necessarily concomitant with a decrease in apolipoprotein(a) mRNA expression, as shown in Example 57.

Example 59

Mismatched and trunctated derivatives of ISIS 301012

As demonstrated herein, ISIS 301012 (SEQ ID NO: 247) reduces apolipoprotein B mRNA levels in cultured human cell lines as well as in human primary hepatocytes. In a further embodiment of the invention, a study was performed using nucleotide sequence derivatives of ISIS 301012. A series of oligonucleotides containing from 1 to 7 base mismatches, starting in the center of the ISIS 301012 sequence, was designed. This series was designed to introduce the consecutive loss of Watson-Crick base pairing between ISIS 301012 and its target mRNA sequence. These compounds are shown in Table 40. The antisense compounds with mismatched nucleotides relative to ISIS 301012 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide.

An additional derivative of ISIS 301012 was designed, comprising the ISIS 301012 sequence with 2'MOE nucleotides throughout the oligonucleotide (uniform 2'-MOE). This compound is 20 nucleotides in length, with phosphorothioate linkages throughout the oligonucleotide. This compound is also shown in Table 40.

HepG2 cells were treated with 50 or 150 nM of the compounds in Table 40 for a 24 hour period, after which RNA was isolated and target expression was measured by real-time PCR as described herein. Untreated cells served as controls. The results are shown in Tables 40 and are normalized to untreated control samples.

Table 40

Effects of ISIS 301012 mismatched oligonucleotides and a uniform 2'MOE oligonucleotide on apolipoprotein B expression in HepG2 cells

ISIS #	SEQUENCE	# Mismatches	% Change in apolipoprotein B mRNA expression Dose of oligonucleotide 50	150	SEQ ID NO
301012	GCCTCAGTCTGCTTCGCACC	0	-44	-75	247
Mismatch Series, chimeric oligonucleotides					
332770	GCCTCAGTCTTCTTCGCACC	1	+7	-22	864
332771	GCCTCAGTCTTATTCGCACC	2	+37	+37	865
332772	GCCTCAGTATTATTCGCACC	3	+99	+84	866
332773	GCCTCATTATTATTCGCACC	4	+75	+80	867
332774	GCCTCATTATTATTAGCACC	5	+62	+66	868
332775	GCCTCATTATTATTATCACC	6	-1	+10	869
332776	GCCTAATTATTATTATCACC	7	+10	+20	870
Uniform 2'-MOE oligonucleotide					
332769	GCCTCAGTCTGCTTCGCACC	0	-11	-14	247

The results of treatment of HepG2 cells with the compounds in Table 40 reveals that none of the compounds displays the dose-dependent inhibition observed following treatment with the parent ISIS 301012 sequence. ISIS 332770, which has only a single thymidine to cytosine substitution in the center of the oligonucleotide, was 3-

fold less potent than ISIS 301012. Further nucleotide substitutions abrogated antisense inhibition of apolipoprotein B expression.

Phosphorothioate chimeric oligonucleotides are metabolized in vivo predominantly by endonucleolytic cleavage. In accordance with the present invention, a series of oligonucleotides was designed by truncating the ISIS 301012 sequence in 1 or 2 base increments from the 5′ and/or 3′ end. The truncated oligonucleotides represent the possible products that result from endonucleotlytic cleavage. These compounds are shown in Table 41. The compounds in Table 41 are chimeric oligonucleotides ("gapmers") of varying lengths, composed of a central "gap" region consisting of 2′-deoxynucleotides, which is flanked on both ends by 2′-methoxyethyl (2′-MOE)nucleotides. The exact structure of each chimeric oligonucleotide is designated in Table 41 as the "chimera structure". For example, a designation of 4~10~4 indicates that the first 4 (5′ most) and last 4 (3′ most) nucleotides are 2′-MOE nucleotides, and the 10 nucleotides in the gap are 2′-deoxynucleotides. 2′-MOE nucleotides are indicated by bold type. The internucleoside (backbone) linkages are phosphodiester (P=O) between underscored nucleotides; all other internucleoside linkages are phosphorothioate (P=S).

These compounds were tested for their ability to reduce the expression of apolipoprotein B mRNA. HepG2 cells were treated with 10, 50 or 150 nM of each antisense compound in Table 41 for a 24 hour period, after which RNA was isolated and target expression was measured by real-time PCR as described herein. Untreated cells served as controls. The results are shown in Tables 41 and are normalized to untreated control samples.

Table 41

Effect of ISIS 301012 truncation mutants on apolipoprotein B expression in HepG2 cells

ISIS #	Target SEQ ID NO	Target Site	SEQUENCE	Chimeric structure	% Change in apolipoprotein B mRNA expression: Dose of oligonucleotide 10	50	150	SEQ ID NO
301012	3	3249	**GCCTC**AGTCTGCTTC**GCACC**	5~10~5	-51	-72	-92	247
331022	3	3249	**GCCTC**AGTCTGCTTC**GCAC**	5~10~4	-33	-49	-87	871
332777	3	3249	**GCCTC**AGTCTGCTTC**GCA**	5~10~3	-27	-53	-80	872
332778	3	3249	**GCCTC**AGTCTGCTTC	5~10~0	-11	-20	-58	873
332780	3	3248	**CCTC**AGTCTGCTTC**GCAC**	4~10~4	-3	-43	-74	874
332781	3	3247	**CTC**AGTCTGCTTC**GCA**	3~10~3	-9	-35	-60	875
332782	3	3246	**TC**AGTCTGCTTC**GC**	2~10~2	-16	-16	-69	876
332784	3	3249	**GCCTC**AGTCT	5~5~0	+12	-1	+7	877
332785	3	3238	GCTTC**GCACC**	0~5~5	+5	-2	-4	878

The results in Table 41 illustrate that inhibition of apolipoprotein B is dependent upon sequence length, as well as upon sequence complementarity and dose, as demonstrated in Table 41, but truncated versions of ISIS 301012 are to a certain degree capable of inhibiting apolipoprotein B mRNA expression.

Example 60

Design and screening of dsRNAs targeting human apolipoprotein B

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements were designed to target apolipoprotein B and are shown in Table 42. All compounds in Table 42 are oligoribonucleotides 20

nucleotides in length with phosphodiester internucleoside linkages (backbones) throughout the compound. The compounds were prepared with blunt ends. Table 41 shows the antisense strand of the dsRNA, and the sense strand is synthesized as the complement of the antisense strand. These sequences are shown to contain uracil (U) but one of skill in the art will appreciate that uracil (U) is generally replaced by thymine (T) in DNA sequences. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the compound binds. A subset of the compounds in Table 42 are the RNA equivalents of DNA antisense oligonucleotides described herein, and, where applicable, this is noted by the ISIS # of the DNA oligonucleotide in the column "RNA equivalent of ISIS #".

Table 42

dsRNAs targeted to human apolipoprotein B

ISIS #	Region	Target SEQ ID NO	Target Site	Sequence	SEQ ID NO	RNA equivalent of ISIS #
342855	coding	3	3249	GCCUCAGUCUGCUUCGCACC	247	301012
342856	3' UTR	3	13903	GCUCACUGUAUGGUUUUAUC	262	301027
342857	coding	3	5589	AGGUUACCAGCCACAUGCAG	224	308361
342858	coding	3	669	GAGCAGUUUCCAUACACGGU	130	270991
342859	coding	3	1179	CCUCUCAGCUCAGUAACCAG	135	270996
342860	coding	3	2331	GUAUAGCCAAAGUGGUCCAC	34	147797
342861	coding	3	3579	UAAGCUGUAGCAGAUGAGUC	213	281614
342862	5' UTR	3	6	CAGCCCCGCAGGUCCCGGUG	249	301014
342863	5' UTR	3	116	GGUCCAUCGCCAGCUGCGGU	256	301021
342864	3' UTR	3	13910	AAGGCUGGCUCACUGUAUGG	266	301031
342865	3' UTR	3	13970	GCCAGCUUUGGUGCAGGUCC	273	301038
342866	coding	3	426	UUGAAGCCAUACACCUCUUU	879	none
342867	coding	3	3001	UGACCAGGACUGCCUGUUCU	880	none
342868	coding	3	5484	GAAUAGGGCUGUAGCUGUAA	881	none
342869	coding	3	6662	UAUACUGAUCAAAUUGUAUC	882	none
342870	coding	3	8334	UGGAAUUCUGGUAUGUGAAG	883	none
342871	coding	3	9621	AAAUCAAAUGAUUGCUUUGU	883	none
342872	coding	3	10155	GUGAUGACACUUGAUUUAAA	885	none
342873	coding	3	12300	GAAGCUGCCUCUUCUUCCCA	886	none

342874	coding	3	13629	GAGAGUUGGUCUGAAAAAUC	887	none

The dsRNA compounds in Table 42 were tested for their effects on human apolipoprotein mRNA in HepG2 cells. HepG2 cells were treated with 100 nM of dsRNA compounds mixed with 5 µg/mL LIPOFECTIN (Invitrogen Corporation, Carlsbad, CA) for a period of 16 hours. In the same experiment, HepG2 cells were also treated with 150 nM of subset of the antisense oligonucleotides described herein mixed with 3.75 µg/mL LIPOFECTIN; these compounds are listed in Table 43. Control oligonucleotides included ISIS 18078 (GTGCGCGCGAGCCCGAAATC, SEQ ID NO: 888). ISIS 18078 is a chimeric oligonucleotide ("gapmer") 20 nucleotides in length, composed of a central "gap" region consisting of 9 2'-deoxynucleotides, which is flanked on the 5' and 3' ends by a five-nucleotide "wing" and a six-nucleotide "wing", respectively. The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidines are 5-methylcytidines. The duplex of ISIS 263188 (CUUCUGGCAUCCGGUUUAGTT, SEQ ID NO: 889) and its complement was also used as a control. ISIS 263188 is an oligoribonucleotide 21 nucleotides in length with the 2 nucleotides on the 3' end being oligodeoxyribonucleotides (TT) and with phosphodiester internucleoside linkages (backbones) throughout the compound.

Cells were treated for 4 hours, after which human apolipoprotein B mRNA expression was measured as described by examples herein. Results were normalized to untreated control cells, which were not treated with LIPOFECTIN or oligonucleotide. Data are the average of 4 experiments and are presented in Table 43.

Table 43

Inhibition of apolipoprotein B mRNA by dsRNAs in HepG2 cells

ISIS #	Dose	% Inhibition	SEQ ID #
342855	100 nM	53	247
342856	100 nM	34	262
342857	100 nM	55	224
342858	100 nM	44	130
342859	100 nM	23	135
342860	100 nM	34	34
342861	100 nM	42	213
342862	100 nM	16	249
342863	100 nM	34	256
342864	100 nM	53	266
342865	100 nM	50	273
342866	100 nM	12	879
342867	100 nM	26	880
342868	100 nM	36	881
342869	100 nM	78	882
342870	100 nM	71	883
342871	100 nM	9	883
342872	100 nM	2	885
342873	100 nM	53	886
342874	100 nM	73	887
281625	150 nM	79	224
301012	150 nM	77	247
301014	150 nM	88	249
301021	150 nM	67	256
301027	150 nM	79	262
301028	150 nM	85	263
301029	150 nM	77	264
301030	150 nM	70	265
301031	150 nM	73	266
301037	150 nM	80	272
301038	150 nM	84	273
301045	150 nM	77	280
263188	150 nM	26	888
18078	150 nM	13	889

Example 61

Antisense inhibition of apolipoprotein B in Cynomolgous monkey primary hepatocytes

As demonstrated in Example 46, the region containing the target site to which ISIS 301012 hybridizes shares 96% identity with the corresponding region of Cynomolgus monkey apolipoprotein B mRNA sequence. ISIS 301012 contains two mismatched nucleotides relative to the Cynomolgous monkey apolipoprotein B mRNA sequence to which it hybridizes. In a further embodiment of the invention, oligonucleotides were designed to target regions of the monkey apolipoprotein B mRNA, using the partial Cynomologous monkey apolipoprotein B sequence described herein (SEQ ID NO: 855) and an additional portion of Cynomolgous monkey apolipoprotein B RNA sequence, incorporated herein as SEQ ID NO: 890. The target site indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. For ISIS 326358 (GCCTCAGTCTGCTTTACACC, SEQ ID NO: 891) the target site is nucleotide 168 of SEQ ID NO: 855 and for ISIS 315089 (AGATTACCAGCCATATGCAG, SEQ ID NO: 892) the target site is nucleotide 19 of SEQ ID NO: 890. ISIS 326358 and ISIS 315089 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. ISIS 326358 and ISIS 315089 are the Cynomolgous monkey equivalents of the human apolipoprotein B antisense oligonucleotides ISIS 301012 (SEQ ID NO: 247) and ISIS 281625 (SEQ ID NO: 224), respectively.

Antisense inhibition by ISIS 301012 was compared to that of ISIS 326358, which is a perfect match to the Cynomolgous monkey apolipoprotein B sequence to which ISIS 301012 hybridizes. The compounds were analyzed for their effect on Cynomolgous monkey apolipoprotein B mRNA levels in primary Cynomolgous monkey hepatocytes purchased from In Vitro Technologies (Gaithersburg, MD). Pre-plated primary Cynonomolgous monkey hepatocytes were purchased from InVitro Technologies (Baltimore, MD). Cells were cultured in high-glucose DMEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA), 100 units/mL and 100 µg/mL streptomycin (Invitrogen Corporation, Carlsbad, CA).

Primary Cynomolgous monkey hepatocytes were treated with 10, 50, 150 or 300 nM of antisense oligonucleotides for 48 hours. ISIS 113529 (SEQ ID NO: 859) was used as a control oligonucleotide. Untreated cells also served as a control. Cynomolgous monkey apolipoprotein B mRNA levels were quantitated by real-time PCR using the human apolipoprotein B and GAPDH primers and probes described by other examples herein. The results, shown in Table 44, are the average of 6 experiments and are expressed as percent inhibition of apolipoprotein B mRNA normalized to untreated control cells.

Table 44

Inhibition of Cynomolgous monkey apolipoprotein B mRNA by ISIS 301012 and ISIS 326358

		% Inhibition of apolipoprotein B mRNA		
		ISIS #		
Dose of oligonucleotide	**Time of treatment**	**326358**	**301012**	**113529**

	(hours)			
10 nM	24	35	24	N.D.
	48	85	76	N.D.
50 nM	24	66	60	N.D.
	48	88	77	N.D.
150 nM	24	61	56	5
	48	82	88	42
300 nM	24	64	61	19
	48	87	86	13

These data demonstrate that both ISIS 326359 and ISIS 301012 (despite two mismatches with the Cynomolgous monkey apolipoprotein B sequence) can inhibit the expression of apolipoprotein B mRNA in cynomolgous monkey primary hepatocytes, in a dose- and time-dependent manner.

Apolipoprotein B protein secreted from primary Cynomolgous hepatocytes treated with 150 and 300 nM of oligonucleotide was measured by ELISA using an apolipoprotein B protein specific kit (ALerCHEK Inc., Portland, ME). Each result represents the average of 3 experiments. The data are normalized to untreated control cells and are shown in Table 45.

Table 45

Reduction in apolipoprotein B protein secreted from Cynomolgous monkey hepatocytes following antisense oligonucleotide treatment

		% Reduction in secreted apolipoprotein B protein		
		ISIS #		
Dose of oligonucleotide	Time of treatment (hours)	326358	301012	113529
150 nM	24	21	31	11
	48	29	25	18
300 nM	24	17	10	12
	48	35	17	8

These results demonstrate that antisense inhibition by ISIS 301012 and ISIS 326358 leads to a decrease in the secretion of apolipoprotein B protein from cultured primary Cynomolgous hepatocytes.

Additionally, protein was isolated from oligonucleotide-treated primary Cynomolgous monkey hepatocytes and subjected to immunoblot analysis to further assess apolipoprotein B protein expression. Immunoblotting was performed as described herein, using an antibody to human apolipoprotein B protein (US Biologicals, Swampscott, MA). Immunoblot analysis of apolipoprotein B expression following antisense oligonucleotide treatment with ISIS 326358 and ISIS 301012 reveals a substantial reduction in apolipoprotein B expression.

In a further embodiment of the invention, antisense inhibition by ISIS 281625 was compared to that by ISIS 315089, which is a perfect match to the Cynomolgous monkey apolipoprotein B sequence to which ISIS 281625 hybridizes. Primary Cynomolgous monkey hepatocytes, cultured as described herein, were treated with 10, 50, 150 or 300 nM of ISIS 315089 or ISIS 281625 for 24 hours. Cells were treated with the control oligonucleotide ISIS 13650 (SEQ ID NO: 806) at 150 and 300 nM or ISIS 113529 (SEQ ID NO: 859) at 300 nM. Untreated cells also served as a control. Cynomolgous monkey apolipoprotein B mRNA levels in primary Cynomolgous monkey hepatocytes was quantitated using real-time PCR with human primers and probe as described by other examples herein. The results, shown in Table 46, are the average of 3 experiments and are expressed as percent inhibition of apolipoprotein B mRNA normalized to untreated control cells. Where present, a "+" preceding the value indicates that mRNA expression was increased.

Table 46

Antisense inhibition of apolipoprotein B mRNA expression in Cynomolgous monkey hepatocytes

	% Inhibition of apolipoprotein B mRNA			
	ISIS #			
Dose of oligonucleotide	315089	281625	13650	113529
10 nM	70	+5	N.D.	N.D.
50 nM	83	41	N.D.	N.D.
150 nM	81	35	+50	N.D.
300 nM	82	69	33	28

These data demonstrate that both ISIS 315089 and ISIS 281625 can inhibit the expression of apolipoprotein B mRNA in Cynomolgous monkey primary hepatocytes, in a dose-dependent manner.

Apolipoprotein B protein secreted primary Cynomolgous hepatocytes treated with 50 and 150 nM of ISIS 315089 and ISIS 281625 was measured by ELISA using an apolipoprotein B protein specific kit (ALerCHEK Inc., Portland, ME). Each result represents the average of 3 experiments. The data are normalized to untreated control cells and are shown in Table 47.

Table 47

Reduction in apolipoprotein B protein secreted from Cynomolgous monkey hepatocytes following antisense oligonucleotide treatment

	% Reduction of monkey apolipoprotein B protein secretion			
	ISIS #			
Dose of oligonucleotide	315089	281625	13650	113529
50 nM	11	6	16	N.D.
150 nM	25	13	13	12

These results demonstrate that antisense inhibition by 150 nM of ISIS 315089 leads to a decrease in the secretion of apolipoprotein B protein from cultured primary Cynomolgous hepatocytes.

ISIS 271009 (SEQ ID NO: 319) and ISIS 301027 (SEQ ID NO: 262) were also tested for their effects on apolipoprotein B mRNA and protein expression in Cynomolgous primary hepatoctyes. Cells, cultured as described herein, were treated with 10, 50 and 150 nM of ISIS 271009 or ISIS 301027 for 24 hours. Cells were treated with the control oligonucleotide ISIS 113529 (SEQ ID NO: 859) at 150 nM. Untreated cells also served as a control. Cynomolgous monkey apolipoprotein B mRNA levels in primary Cynomolgous monkey hepatocytes was quantitated using real-time PCR with human primers and probe as described by other examples herein. The results, shown in Table 48, are the average of 2 experiments and are expressed as percent inhibition of apolipoprotein B mRNA normalized to untreated control cells.

Table 48

Antisense inhibition of apolipoprotein B mRNA expression in Cynomolgous monkey hepatocytes

	% Inhibition of apolipoprotein B mRNA		
	ISIS #		
Dose of oligonucleotide	**271009**	**301027**	**113529**
10 nM	42	40	N.D.
50 nM	66	54	N.D.
150 nM	69	67	11

These data demonstrate that both ISIS 271009 and ISIS 301027 can inhibit the expression of apolipoprotein B mRNA

in Cynomolgous monkey primary hepatocytes, in a dose-dependent manner.

Apolipoprotein B protein secreted from primary Cynomolgous hepatocytes treated with 50 and 150 nM of ISIS 271009 and ISIS 301027 was measured by ELISA using an apolipoprotein B protein specific kit (ALerCHEK Inc., Portland, ME). Each result represents the average of 3 experiments. The data are shown as percent reduction in secreted protein, normalized to untreated control cells, and are shown in Table 49. Where present, a "+" indicates that protein secretion was increased.

Table 49

Reduction in apolipoprotein B protein secreted from Cynomolgous monkey hepatocytes following antisense oligonucleotide treatment

	% Reduction of monkey apolipoprotein B protein secretion			
	ISIS #			
Dose of oligonucleotide	**271009**	**301027**	**13650**	**113529**
50 nM	+30	25	N.D.	N.D.
150 nM	26	31	+1	15

These results demonstrate that antisense inhibition by ISIS 315089 and ISIS 281625 leads to a decrease in the secretion of apolipoprotein B protein from cultured primary Cynomolgous hepatocytes.

Example 62

Methods for evaluating hepatic steatosis

Hepatic steatosis refers to the accumulation of lipids in the liver, or "fatty liver", which is frequently caused

by alcohol consumption, diabetes and hyperlipidemia. Livers of animals treated with antisense oligonucleotides targeted to apolipoprotein B were evaluated for the presence of steatosis. Steatosis is assessed by histological analysis of liver tissue and measurement of liver triglyceride levels.

Tissue resected from liver is immediately immersed in Tissue Tek OCT embedding compound (Ted Pella, Inc., Redding, CA) and frozen in a 2-methyl-butane dry ice slurry. Tissue sections are cut at a thickness of 4-5 µm and then fixed in 5% neutral-buffered formalin. Tissue sections are stained with hematoxylin and eosin following standard histological procedures to visualize nuclei and cytoplasm, respectively, and oil red O according to the manufacturer's instructions (Newcomers Supply, Middleton, WI) to visualize lipids.

Alternatively, tissues are fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at a thickness of 4-5 µm, deparaffinized and stained with hematoxylin and eosin, all according to standard histological procedures.

Quantitation of liver triglyceride content is also used to assess steatosis. Tissue triglyceride levels are measured using a Triglyceride GPO Assay (Sigma-Aldrich, St. Louis, MO).

Example 63

Effects of antisense inhibition by ISIS 301012 in lean mice: long-term study

In accordance with the present invention, the toxicity of ISIS 301012 (SEQ ID NO: 247) is investigated in a long-term, 3 month study in mice. Two-month old male and

female CD-1 mice (Charles River Laboratories, Wilmington, MA) are dosed with 2, 5, 12.5, 25 or 50 mg/kg of ISIS 301012 twice per week for first week, and every 4 days thereafter. The mice are maintained on a standard rodent diet. Saline and control oligonucleotide animals serve as controls and are injected on the same schedule. Each treatment group contains 6 to 10 mice of each sex, and each treatment group is duplicated, one group for a 1 month study termination, the other for a 3 month study termination. After the 1 or 3 month treatment periods, the mice are sacrificed and evaluated for target expression in liver, lipid levels in serum and indicators of toxicity. Liver samples are procured, RNA is isolated and apolipoprotein B mRNA expression is measured by real-time PCR as described in other examples herein. Serum lipids, including total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides, are evaluated by routine clinical analysis using an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY). Ratios of LDL-cholesterol to HDL-cholesterol and total cholesterol to HDL-cholesterol are also calculated. Analyses of serum ALT and AST, inflammatory infiltrates in tissue and basophilic granules in tissue provide an assessment of toxicities related to the treatment. Hepatic steatosis, or accumulation of lipids in the liver, is assessed by routine histological analysis with oil red O stain and measurement of liver tissue triglycerides using a Triglyceride GPO Assay (Sigma-Aldrich, St. Louis, MO).

The toxicity study also includes groups of animals allowed to recover following cessation of oligonucleotide treatment. Both male and female CD-1 mice (Charles River Laboratories, Wilmington, MA) are treated with 5, 10, 50

mg/kg of ISIS 301012 twice per week for the first week and every 4 days thereafter. Saline and control oligonucleotide injected animals serve as controls. Each treatment group includes 6 animals per sex. After 3 months of treatment, animals remain untreated for an additional 3 months, after which they are sacrificed. The same parameters are evaluated as in the mice sacrificed immediately after 3 months of treatment.

After one month of treatment, real-time PCR quantitation reveals that mouse apolipoprotein B mRNA levels in liver are reduced by 53%. Additionally, the expected dose-response toxicities were observed. ALT and AST levels, measured by routine clinical procedures on an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY), are increased in mice treated with 25 or 50 mg/kg of ISIS 301012. Tissues were prepared for analysis by routine histological procedures. Basophilic granules in liver and kidney tissue were observed at doses of ISIS 301012 above 12.5 mg/kg. Mild lymphohistiocytic infiltrates were observed in various tissues at doses greater than 12.5 mg/kg of ISIS 301012. Staining of tissue sections with oil red O reveals no steatosis present following the oligonucleotide treatments.

Example 64

Effects of antisense inhibition by ISIS 301012 in lean Cynomolgous monkeys: long-term study

As discussed in Example 45, Cynomolgus monkeys (male or female) are used to evaluate antisense oligonucleotides for their potential to lower apolipoprotein B mRNA or protein levels, as well as phenotypic endpoints associated with apolipoprotein B including, but not limited to

cardiovascular indicators, atherosclerosis, lipid diseases, obesity, and plaque formation. Accordingly, in a further embodiment of the invention, ISIS 301012 (SEQ ID NO: 247) is investigated in a long-term study for its effects on apolipoprotein B expression and serum lipids in Cynomolgous monkeys. Such a long-term study is also used to evaluate the toxicity of antisense compounds.

Male and female Cynomologous monkeys are treated with 2, 4 or 12 mg/kg of ISIS 301012 intravenously or 2 or 20 mg/kg subcutaneously at a frequency of every two days for the first week, and every 4 days thereafter, for 1 and 3 month treatment periods. Saline-treated animals serve as controls. Each treatment group includes 2 to 3 animals of each sex.

At a one month interval and at the 3 month study termination, the animals are sacrificed and evaluated for target expression in liver, lipid levels in serum and indicators of toxicity. Liver samples are procured, RNA is isolated and apolipoprotein B mRNA expression is measured by real-time PCR as described in other examples herein. Serum lipids, including total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides, are evaluated by routine clinical analysis using an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY). Ratios of LDL-cholesterol to HDL-cholesterol and total cholesterol to HDL-cholesterol are also calculated. Analyses of serum ALT and AST, inflammatory infiltrates in tissue and basophilic granules in tissue provide an assessment of toxicities related to the treatment. Hepatic steatosis, or accumulation of lipids in the liver, is assessed by routine histological analysis with oil red O stain and measurement

of liver tissue triglycerides using a Triglyceride GPO Assay (Sigma-Aldrich, St. Louis, MO).

Additional treatment groups consisting of 2 animals per sex are treated with saline (0 mg/kg), 12 or 20 mg/kg ISIS 301012 at a frequency of every two days for the first week, and every 4 days thereafter, for a 3 month period. Following the treatment period, the animals receive no treatment for an additional three months. These treatment groups are for the purpose of studying the effects of apolipoprotein B inhibition 3 months after cessation of treatment. At the end of the 3 month recovery period, animals are sacrificed and evaluated for the same parameters as the animals sacrificed immediately after 1 and 3 months of treatment.

The results from the one month interval of the long term treatment are shown in Table 50 and are normalized to saline-treated animals for mRNA and to untreated baseline values for lipid levels. Total cholesterol, LDL-cholesterol, HDL-cholesterol, LDL particle concentration and triglyceride levels in serum were measured by nuclear magnetic resonance spectroscopy by Liposcience (Raleigh, NC). Additionally, the concentration of intact oligonucleotide in liver was measured by capillary gel electrophoresis and is presented as micrograms of oligonucleotide per gram of liver tissue. Each result represents the average of data from 4 animals (2 males and 2 females).

Table 50

Effects of antisense inhibition by ISIS 301012 in lean Cynomolgous monkeys

		Intravenous delivery			Subcutaneous injection	
		2 mg/kg	4 mg/kg	12 mg/kg	3.5 mg/kg	20 mg/kg
apolipoprotein B expression % change normalized to saline		-45	-76	-96	N.D.	-94
antisense oligonucleotide concentration µg/g		92	179	550	N.D.	855
Lipid parameters, % change normalized to untreated baseline value	Saline	2 mg/kg	4 mg/kg	12 mg/kg	3.5 mg/kg	20 mg/kg
Total cholesterol	+1	-6	-2	-2	+5	-5
LDL-cholesterol	+17	+15	+9	+3	-4	-16
HDL-cholesterol	-11	-23	-15	-8	+13	+5
LDL/HDL	+62	+94	+38	+44	-15	-19
Total cholesterol/HDL	+30	+44	+22	+21	-7	-10
Triglyceride	+37	+26	+32	+15	+1	-3
LDL Particle concentration	+15	+8	+8	-11	-14	-21

These data show that ISIS 301012 inhibits apolipoprotein B expression in a dose-dependent manner in a primate species and concomitantly lowers lipid levels at higher doses of ISIS 301012. Furthermore, these results demonstrate that antisense oligonucleotide accumulates in the liver in a dose-dependent manner.

Hepatic steatosis, or accumulation of lipids in the liver, was not observed following 4 weeks of treatment with the doses indicated. Expected dose-related toxicities were observed at the higher doses of 12 and 20 mg/kg, including a transient 1.2-1.3 fold increase in activated partial thromboplastin time (APTT) during the first 4 hours and basophilic granules in the liver and kidney (as assessed by routine histological examination of tissue samples). No functional changes in kidney were observed.

In a similar experiment, male and female Cynomolgous monkeys received an intravenous dose of ISIS 301012 at 4 mg/kg, every two days for the first week and every 4 days thereafter. Groups of animals were sacrificed after the

first dose and the fourth dose, as well as 11, 15 and 23 days following the fourth and final dose. Liver RNA was isolated and apolipoprotein B mRNA levels were evaluated by real-time PCR as described herein. The results of this experiment demonstrate a 40% reduction in apolipoprotein B mRNA expression after a single intravenous dose of 4 mg/kg ISIS 301012. Furthermore, after 4 doses of ISIS 301012 at 4 mg/kg, target mRNA was reduced by approximately 85% and a 50% reduction in target mRNA was sustained for up to 16 days following the cessation of antisense oligonucleotide treatment.

Example 65

Microarray analysis: gene expression patterns in lean versus high-fat fed mice

Male C57Bl/6 mice were divided into the following groups, consisting of 5 animals each: (1) mice on a lean diet, injected with saline (lean control); (2) mice on a high fat diet; (3) mice on a high fat diet injected with 50 mg/kg of the control oligonucleotide 141923 (SEQ ID NO: 858); (4) mice on a high fat diet given 20 mg/kg atorvastatin calcium (Lipitor®, Pfizer Inc.); (5) mice on a high fat diet injected with 10, 25 or 50 mg/kg ISIS 147764 (SEQ ID NO: 109). Saline and oligonucleotide treatments were administered intraperitoneally twice weekly for 6 weeks. Atorvastatin was administered daily for 6 weeks. At study termination, liver samples were isolated from each animal and RNA was isolated for Northern blot qualitative assessment, DNA microarray and quantitative real-time PCR. Northern blot assessment and quantitative real-time PCR were performed as described by other examples herein.

For DNA microarray analysis, hybridization samples

were prepared from 10 µg of total RNA isolated from each mouse liver according to the Affymetrix Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA). Samples were hybridized to a mouse gene chip containing approximately 22,000 genes, which was subsequently washed and double-stained using the Fluidics Station 400 (Affymetrix, Inc., Santa Clara, CA) as defined by the manufacturer's protocol. Stained gene chips were scanned for probe cell intensity with the GeneArray scanner (Affymetrix, Inc., Santa Clara, CA). Signal values for each probe set were calculated using the Affymetrix Microarray Suite v5.0 software (Affymetrix, Inc., Santa Clara, CA). Each condition was profiled from 5 biological samples per group, one chip per sample. Fold change in expression was computed using the geometric mean of signal values as generated by Microarray Suite v5.0. Statistical analysis utilized one-way ANOVA followed by 9 pair-wise comparisons. All groups were compared to the high fat group to determine gene expression changes resulting from ISIS 147764 treatment. Microarray data was interpreted using hierarchical clustering to visualize global gene expression patterns.

The results of the microarray analysis reveal that treatment with ISIS 147764 drives the gene expression profile in high fat fed mice to the profile observed in lean mice. Real-time PCR analysis confirmed the reduction in mRNA expression for the following genes involved in the lipid metabolism: hepatic lipase, fatty acid synthase ATP-binding cassette, sub-family D (ALD) member 2, intestinal fatty acid binding protein 2, stearol CoA desaturase-1 and HMG CoA reductase.

Mouse apolipoprotein B mRNA and serum cholesterol levels, measured as described herein, were evaluated to confirm antisense inhibition by ISIS 147764 and ISIS 147483. Both mRNA and cholesterol levels were lowered in a dose-dependent manner following treatment with ISIS 147764 or ISIS 147483, as demonstrated in other examples herein. The 50 mg/kg dose of ISIS 147483 increased ALT and AST levels. The 10, 25 and 50 mg/kg doses of ISIS 147764 and the 10 and 25 mg/kg doses of ISIS 147483 did not significantly elevate ALT or AST levels.

Example 66

Evaluation of hepatic steatosis in animals treated with apolipoprotein B antisense oligonucleotides

Livers of animals treated with antisense oligonucleotides targeted to apolipoprotein B were evaluated for the presence of steatosis. Steatosis is assessed by histological analysis of liver tissue and measurement of liver triglyceride levels.

Evaluation of steatosis in high fat fed animals treated with ISIS 147764 for 6 weeks

Liver tissue from ISIS 147764 (SEQ ID NO: 109) and control-treated animals described in Example 21 was evaluated for steatosis at study termination following 6 weeks of treatment. Tissue sections were stained with oil red O and hematoxylin to visualize lipids and nuclei, respectively. Tissue sections were also stained with hematoxylin and eosin to visualize nuclei and cytoplasm, respectively. Histological analysis of tissue sections stained by either method reveal no difference in steatosis between saline treated and ISIS 147764 treated animals,

demonstrating that a 6 week treatment with ISIS 147764 does not lead to accumulation of lipids in the liver.

Evaluation of steatosis following long-term treatment with apolipoprotein B inhibitor in high-fat fed animals

Male C57Bl/6 mice were treated with twice weekly intraperitoneal injections of 25 mg/kg ISIS 147764 (SEQ ID NO: 109) or 25 mg/kg ISIS 141923 (SEQ ID NO: 858) for 6, 12 and 20 weeks. Saline treated animals served as controls. Each treatment group contained 4 animals. Animals were sacrificed at 6, 12 and 20 weeks and liver tissue was procured for histological analysis and measurement of tissue triglyeride content. The results reveal no significant differences in liver tissue triglyceride content when ISIS 147764 treated animals are compared to saline treated animals. Furthermore, histological analysis of liver tissue section demonstrates that steatosis is reduced at 12 and 20 weeks following treatment of high fat fed mice with ISIS 147764, in comparison to saline control animals that received a high fat diet.

Evaluation of steatosis in lean mice

The accumulation of lipids in liver tissue was also evaluated in lean mice. Male C67Bl/6 mice (Charles River Laboratories (Wilmington, MA) at 6 to 7 weeks of age were maintained on a standard rodent diet and were treated twice weekly with intraperitoneal injections of 25 or 50 mg/kg 147764 (SEQ ID NO: 109) or 147483 (SEQ ID NO: 79) for 6 weeks. Saline treated animals served as controls. Each treatment group was comprised of 4 animals. Animals were sacrificed after the 6 week treatment period, at which point liver tissue and serum were collected.

Apolipoprotein B mRNA levels were measured by real-time PCR as described by other examples herein. The data, shown in Table 51, represent the average of 4 animals and are presented as inhibition relative to saline treated controls. The results demonstrate that both ISIS 147483 and ISIS 147764 inhibit apolipoprotein B mRNA expression in lean mice in a dose-dependent manner.

Table 51

Antisense inhibition of apolipoprotein B mRNA in lean mice

	Treatment and dose			
	ISIS 147483		ISIS 147764	
	25 mg/kg	50 mg/kg	25 mg/kg	50 mg/kg
% inhibition apolipoprotein B mRNA	79	91	48	77

Total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides in serum were measured by routine clinical analysis using an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY). The liver enzymes ALT and ALT in serum were also measured using the Olympus Clinical Analyzer. These results demonstrate that ISIS 147764 lowers serum lipids relative to saline-treated control animals. ALT and AST levels do not exceed the normal range for mice (300 IU/L), indicating a lack of treatment-associated toxicity. The results are the average of data from 4 animals and are shown in Table 52.

Table 52

Serum lipids and liver enzyme levels in lean mice treated with ISIS 147764 and ISIS 147483

	Treatment and dose				
	Saline	ISIS 147483		ISIS 147764	
		25 mg/kg	50 mg/kg	25 mg/kg	50 mg/kg
Serum lipids					
Total cholesterol mg/dL	164	153	183	114	57
LDL-cholesterol mg/dL	25	26	39	29	18
HDL-cholesterol mg/dL	127	117	131	79	38
Triglycerides mg/dL	121	138	127	80	30
Liver enzymes					
ALT IU/L	105	73	57	47	48
AST IU/L	109	78	72	81	101

Liver tissue was prepared by routine histological methods to evaluate steatosis, as described herein. Examination of tissue samples stained with oil red O or hematoxylin and eosin reveals that treatment of lean mice with apolipoprotein B antisense oligonucleotides does not result in steatosis.

Six month study to further evaluate steatosis in mice treated with apolipoprotein B antisense oligonucleotides

A long-term treatment of mice with antisense oligonucleotides targeted to apolipoprotein B is used to evaluate the toxicological and pharmacological effects of extended treatment with antisense compounds. Both male and female C57Bl/6 mice at 2 months of age are treated with 2, 5, 25 or 50 mg/kg of apolipoprotein B antisense oligonucleotide. Treatments are administered intraperitoneally every 2 days for the first week and every 4 days thereafter. Mice treated with saline alone or control oligonucleotide serve as control groups. Each

treatment group contains 25 to 30 mice. After 6 months of treatment, a subset of the mice in each treatment group is sacrificed. The remaining mice are allowed a 3 month recovery period without treatment, after which they are sacrificed. Apolipoprotein B mRNA expression in liver is measured by real-time PCR as described by other methods herein. Liver tissue is also prepared for measurement of triglyceride content using a Triglyceride GPO Assay (Sigma-Aldrich, St. Louis, MO). Serum is collected and evaluated for lipid content, including total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride, using an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY). The liver enzymes ALT and AST are also measured in serum, also using the clinical analyzer. Serum samples are subjected to immunoblot analysis using an antibody directed to apolipoprotein B (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Liver, kidney and other tissues are prepared by routine procedures for histological analyses. Tissues are evaluated for the presence of basophilic granules and inflammatory infiltrates. Steatosis is evaluated by oil red O stain of liver tissue sections.

Example 67

A mouse model for atherosclerotic plaque formation: human apolipoprotein B transgenic mice lacking the LDL receptor gene

The LDL receptor is responsible for clearing apolipoprotein B-containing LDL particles. Without the LDL receptor, animals cannot effectively clear apolipoprotein B-containing LDL particles from the plasma. Thus the serum levels of apolipoprotein B and LDL cholesterol are markedly elevated. Mice expressing the human apolipoprotein B

transgene (TgN-hApoB +/+) and mice deficient for the LDL receptor (LDLr -/-) are both used as animal models of atherosclerotic plaque development. When the LDL receptor deficiency genotype is combined with a human apolipoprotein B transgenic genotype (TgN-hApoB +/+; LDLr -/-), atherosclerotic plaques develop rapidly. In accordance with the present invention, mice of this genetic background are used to investigate the ability of compounds to prevent atherosclerosis and plaque formation.

Male TgN-hApoB +/+;LDLr -/- mice are treated twice weekly with 10 or 20 mg/kg of human apolipoprotein B antisense oligonucleotides for 12 weeks. Control groups are treated with saline or control oligonucleotide. Serum total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides are measured at 2, 4, 6, 8 and 12 weeks by routine clinical analysis using an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY). Serum human apolipoprotein B protein is measured at 2, 4, 6, 8 and 12 weeks using an ELISA kit (ALerCHEK Inc., Portland, ME). Human and mouse apolipoprotein mRNA in liver is measured at 12 weeks. The results of the 12 week study serve to evaluate the pharmacological behavior of ISIS 301012 in a doubly transgenic model.

Additionally, a four month study is performed in TgN-hApoB +/+;LDLr -/- mice, with treatment conditions used in the 12 week study. Mice are treated for 4 months with antisense oligonucleotides targeted to human apolipoprotein B to evaluate the ability of such compounds to prevent atherosclerotic plaque formation. At the end of the 4 month treatment period, mice are anesthetized and perfused with 10% formalin. The perfused arterial tree is isolated and examined for the presence of atherosclerotic plaques.

Sections of the arterial tree are embedded in paraffin and prepared for histological analysis using routine methods. Serum total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides are measured at 2, 4, 6, 8, 12 and 16 weeks by routine clinical analysis using an Olympus Clinical Analyzer (Olympus America Inc., Melville, NY). Serum human apolipoprotein B protein is measured at 2, 4, 6, 8, 12 and 16 weeks using an ELISA kit (ALerCHEK Inc., Portland, ME). Human and mouse apolipoprotein mRNA in liver at 16 weeks is measured by real-time PCR.

Example 68

Rabbit models for study of atherosclerotic plaque formation

The Watanabe heritable hyperlipidemic (WHHL) strain of rabbit is used as a model for atherosclerotic plaque formation. New Zealand white rabbits on a high-fat diet are also used as a model of atherosclerotic plaque formation. Treatment of WHHL or high fat fed New Zealand white rabbits with apolipoprotein B antisense compounds is used to test their potential as therapeutic or prophylactic treatments for atherosclerotic plaque disease. Rabbits are injected with 5, 10, 25 or 50 mg/kg of antisense oligonucleotides targeted to apolipoprotein B. Animals treated with saline alone or a control oligonucleotide serve as controls. Throughout the treatment, serum samples are collected and evaluated for apolipoprotein B protein levels by ELISA (kit from ALerCHEK Inc., Portland, ME) and serum lipids (cholesterol, LDL-cholesterol, VLDL-cholesterol, HDL-cholesterol, triglycerides) by routine clinical analysis. Liver tissue triglyceride content is measured using a Triglyceride GPO Assay (Sigma-Aldrich, St. Louis, MO). Liver, kidney, heart, aorta and other tissues

are procured and processed for histological analysis using routine procedures. Liver and kidney tissues are examined for evidence of basophilic granules and inflammatory infiltrates. Liver tissue is evaluated for steatosis using oil red O stain. Additionally, aortic sections stained with oil red O stain and hematoxylin are examined to evaluate the formation of atherosclerotic lesions.

Example 69

Oral delivery of apolipoprotein B inhibitors

Oligonucleotides may be formulated for delivery *in vivo* in an acceptable dosage form, e.g. as parenteral or non-parenteral formulations. Parenteral formulations include intravenous (IV), subcutaneous (SC), intraperitoneal (IP), intravitreal and intramuscular (IM) formulations, as well as formulations for delivery via pulmonary inhalation, intranasal administration, topical administration, etc. Non-parenteral formulations include formulations for delivery via the alimentary canal, e.g. oral administration, rectal administration, intrajejunal instillation, etc. Rectal administration includes administration as an enema or a suppository. Oral administration includes administration as a capsule, a gel capsule, a pill, an elixir, etc.

In some embodiments, an oligonucleotide may be administered to a subject via an oral route of administration. The subject may be an animal or a human (man). An animal subject may be a mammal, such as a mouse, rat, mouse, a rat, a dog, a guinea pig, a monkey, a non-human primate, a cat or a pig. Non-human primates include monkeys and chimpanzees. A suitable animal subject may be an experimental animal, such as a mouse, rat, mouse, a rat, a dog, a monkey, a non-human primate, a cat or a pig.

In some embodiments, the subject may be a human. In certain embodiments, the subject may be a human patient in

need of therapeutic treatment as discussed in more detail herein. In certain embodiments, the subject may be in need of modulation of expression of one or more genes as discussed in more detail herein. In some particular embodiments, the subject may be in need of inhibition of expression of one or more genes as discussed in more detail herein. In particular embodiments, the subject may be in need of modulation, i.e. inhibition or enhancement, of apolipoprotein B in order to obtain therapeutic indications discussed in more detail herein.

In some embodiments, non-parenteral (e.g. oral) oligonucleotide formulations according to the present invention result in enhanced bioavailability of the oligonucleotide. In this context, the term "bioavailability" refers to a measurement of that portion of an administered drug which reaches the circulatory system (e.g. blood, especially blood plasma) when a particular mode of administration is used to deliver the drug. Enhanced bioavailability refers to a particular mode of administration's ability to deliver oligonucleotide to the peripheral blood plasma of a subject relative to another mode of administration. For example, when a non-parenteral mode of administration (e.g. an oral mode) is used to introduce the drug into a subject, the bioavailability for that mode of administration may be compared to a different mode of administration, e.g. an IV mode of administration. In some embodiments, the area under a compound's blood plasma concentration curve (AUC_0) after non-parenteral (e.g. oral, rectal, intrajejunal) administration may be divided by the area under the drug's plasma concentration curve after intravenous (i.v.) administration (AUC_{iv}) to provide a dimensionless quotient (relative bioavailability, RB) that represents fraction of compound absorbed via the non-parenteral route as compared to the IV route. A composition's bioavailability is said to be enhanced in comparison to another composition's

bioavailability when the first composition's relative bioavailability (RB_1) is greater than the second composition's relative bioavailability (RB_2).

In general, bioavailability correlates with therapeutic efficacy when a compound's therapeutic efficacy is related to the blood concentration achieved, even if the drug's ultimate site of action is intracellular (van Berge-Henegouwen *et al.*, *Gastroenterol.*, 1977, *73,* 300). Bioavailability studies have been used to determine the degree of intestinal absorption of a drug by measuring the change in peripheral blood levels of the drug after an oral dose (DiSanto, Chapter 76 *In: Remington=s Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 1451-1458).

In general, an oral composition's bioavailability is said to be "enhanced" when its relative bioavailability is greater than the bioavailability of a composition substantially consisting of pure oligonucleotide, i.e. oligonucleotide in the absence of a penetration enhancer.

Organ bioavailability refers to the concentration of compound in an organ. Organ bioavailability may be measured in test subjects by a number of means, such as by whole-body radiography. Organ bioavailability may be modified, e.g. enhanced, by one or more modifications to the oligonucleotide, by use of one or more carrier compounds or excipients, etc. as discussed in more detail herein. In general, an increase in bioavailability will result in an increase in organ bioavailability.

Oral oligonucleotide compositions according to the present invention may comprise one or more "mucosal penetration enhancers," also known as "absorption enhancers" or simply as "penetration enhancers." Accordingly, some embodiments of the invention comprise at least one oligonucleotide in combination with at least one penetration enhancer. In general, a penetration enhancer is a substance that facilitates the transport of a drug across mucous membrane(s) associated with the desired mode

of administration, e.g. intestinal epithelial membranes. Accordingly it is desirable to select one or more penetration enhancers that facilitate the uptake of an oligonucleotide, without interfering with the activity of the oligonucleotide, and in a such a manner the oligonucleotide can be introduced into the body of an animal without unacceptable side-effects such as toxicity, irritation or allergic response.

Embodiments of the present invention provide compositions comprising one or more pharmaceutically acceptable penetration enhancers, and methods of using such compositions, which result in the improved bioavailability of oligonucleotides administered via non-parenteral modes of administration. Heretofore, certain penetration enhancers have been used to improve the bioavailability of certain drugs. See Muranishi, *Crit. Rev. Ther. Drug Carrier Systems*, 1990, 7, 1 and Lee *et al.*, *Crit. Rev. Ther. Drug Carrier Systems*, 1991, *8*, 91. It has been found that the uptake and delivery of oligonucleotides, relatively complex molecules which are known to be difficult to administer to animals and man, can be greatly improved even when administered by non-parenteral means through the use of a number of different classes of penetration enhancers.

In some embodiments, compositions for non-parenteral administration include one or more modifications from naturally-occurring oligonucleotides (i.e. full-phosphodiester deoxyribosyl or full-phosphodiester ribosyl oligonucleotides). Such modifications may increase binding affinity, nuclease stability, cell or tissue permeability, tissue distribution, or other biological or pharmacokinetic property. Modifications may be made to the base, the linker, or the sugar, in general, as discussed in more detail herein with regards to oligonucleotide chemistry. In some embodiments of the invention, compositions for administration to a subject, and in particular oral compositions for administration to an animal or human

subject, will comprise modified oligonucleotides having one or more modifications for enhancing affinity, stability, tissue distribution, or other biological property.

Suitable modified linkers include phosphorothioate linkers. In some embodiments according to the invention, the oligonucleotide has at least one phosphorothioate linker. Phosphorothioate linkers provide nuclease stability as well as plasma protein binding characteristics to the oligonucleotide. Nuclease stability is useful for increasing the *in vivo* lifetime of oligonucleotides, while plasma protein binding decreases the rate of first pass clearance of oligonucleotide via renal excretion. In some embodiments according to the present invention, the oligonucleotide has at least two phosphorothioate linkers. In some embodiments, wherein the oligonucleotide has exactly n nucleosides, the oligonucleotide has from one to n-1 phosphorothioate linkages. In some embodiments, wherein the oligonucleotide has exactly n nucleosides, the oligonucleotide has n-1 phosphorothioate linkages. In other embodiments wherein the oligonucleotide has exactly n nucleoside, and n is even, the oligonucleotide has from 1 to n/2 phosphorothioate linkages, or, when n is odd, from 1 to (n-1)/2 phosphorothioate linkages. In some embodiments, the oligonucleotide has alternating phosphodiester (PO) and phosphorothioate (PS) linkages. In other embodiments, the oligonucleotide has at least one stretch of two or more consecutive PO linkages and at least one stretch of two or more PS linkages. In other embodiments, the oligonucleotide has at least two stretches of PO linkages interrupted by at least on PS linkage.

In some embodiments, at least one of the nucleosides is modified on the ribosyl sugar unit by a modification that imparts nuclease stability, binding affinity or some other beneficial biological property to the sugar. In some cases, the sugar modification includes a 2'-modification, e.g. the 2'-OH of the ribosyl sugar is replaced or substituted. Suitable replacements for 2'-OH include 2'-F

and 2'-arabino-F. Suitable substitutions for OH include 2'-*O*-alkyl, e.g. 2-*O*-methyl, and 2'-*O*-substituted alkyl, e.g. 2'-*O*-methoxyethyl, 2'-*O*-aminopropyl, etc. In some embodiments, the oligonucleotide contains at least one 2'-modification. In some embodiments, the oligonucleotide contains at least 2 2'-modifications. In some embodiments, the oligonucleotide has at least one 2'-modification at each of the termini (i.e. the 3'- and 5'-terminal nucleosides each have the same or different 2'-modifications). In some embodiments, the oligonucleotide has at least two sequential 2'-modifications at each end of the oligonucleotide. In some embodiments, oligonucleotides further comprise at least one deoxynucleoside. In particular embodiments, oligonucleotides comprise a stretch of deoxynucleosides such that the stretch is capable of activating RNase (e.g. RNase H) cleavage of an RNA to which the oligonucleotide is capable of hybridizing. In some embodiments, a stretch of deoxynucleosides capable of activating RNase-mediated cleavage of RNA comprises about 6 to about 16, e.g. about 8 to about 16 consecutive deoxynucleosides.

Oral compositions for administration of non-parenteral oligonucleotide compositions of the present invention may be formulated in various dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The term "alimentary delivery" encompasses e.g. oral, rectal, endoscopic and sublingual/buccal administration. A common requirement for these modes of administration is absorption over some portion or all of the alimentary tract and a need for efficient mucosal penetration of the nucleic acid(s) so administered.

Delivery of a drug via the oral mucosa, as in the case of buccal and sublingual administration, has several desirable features, including, in many instances, a more rapid rise in plasma concentration of the drug than via oral delivery (Harvey, Chapter 35 *In: Remington=s*

Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, page 711).

Endoscopy may be used for drug delivery directly to an interior portion of the alimentary tract. For example, endoscopic retrograde cystopancreatography (ERCP) takes advantage of extended gastroscopy and permits selective access to the biliary tract and the pancreatic duct (Hirahata *et al.*, *Gan To Kagaku Ryoho*, 1992, *19(10 Suppl.)*, 1591). Pharmaceutical compositions, including liposomal formulations, can be delivered directly into portions of the alimentary canal, such as, *e.g.*, the duodenum (Somogyi *et al.*, *Pharm. Res.*, 1995, *12*, 149) or the gastric submucosa (Akamo *et al.*, *Japanese J. Cancer Res.*, 1994, *85*, 652) via endoscopic means. Gastric lavage devices (Inoue *et al.*, *Artif. Organs*, 1997, *21*, 28) and percutaneous endoscopic feeding devices (Pennington *et al.*, *Ailment Pharmacol. Ther.*, 1995, *9*, 471) can also be used for direct alimentary delivery of pharmaceutical compositions.

In some embodiments, oligonucleotide formulations may be administered through the anus into the rectum or lower intestine. Rectal suppositories, retention enemas or rectal catheters can be used for this purpose and may be preferred when patient compliance might otherwise be difficult to achieve (*e.g.*, in pediatric and geriatric applications, or when the patient is vomiting or unconscious). Rectal administration can result in more prompt and higher blood levels than the oral route. (Harvey, Chapter 35 *In: Remington=s Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, page 711). Because about 50% of the drug that is absorbed from the rectum will bypass the liver, administration by this route significantly reduces the potential for first-pass metabolism (Benet *et al.*, Chapter 1 *In: Goodman & Gilman=s The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996).

One advantageous method of non-parenteral administration oligonucleotide compositions is oral delivery. Some embodiments employ various penetration enhancers in order to effect transport of oligonucleotides and other nucleic acids across mucosal and epithelial membranes. Penetration enhancers may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Accordingly, some embodiments comprise oral oligonucleotide compositions comprising at least one member of the group consisting of surfactants, fatty acids, bile salts, chelating agents, and non-chelating surfactants. Further embodiments comprise oral oligonucleotide comprising at least one fatty acid, e.g. capric or lauric acid, or combinations or salts thereof. Other embodiments comprise methods of enhancing the oral bioavailability of an oligonucleotide, the method comprising co-administering the oligonucleotide and at least one penetration enhancer.

Other excipients that may be added to oral oligonucleotide compositions include surfactants (or "surface-active agents"), which are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the alimentary mucosa and other epithelial membranes is enhanced. In addition to bile salts and fatty acids, surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and perfluorohemical emulsions, such as FC-43 (Takahashi *et al.*, *J. Pharm. Phamacol.*, 1988, *40*, 252).

Fatty acids and their derivatives which act as penetration enhancers and may be used in compositions of

the present invention include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines and mono- and di-glycerides thereof and/or physiologically acceptable salts thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1; El-Hariri *et al.*, *J. Pharm. Pharmacol.*, 1992, *44*, 651).

In some embodiments, oligonucleotide compositions for oral delivery comprise at least two discrete phases, which phases may comprise particles, capsules, gel-capsules, microspheres, etc. Each phase may contain one or more oligonucleotides, penetration enhancers, surfactants, bioadhesives, effervescent agents, or other adjuvant, excipient or diluent. In some embodiments, one phase comprises at least one oligonucleotide and at lease one penetration enhancer. In some embodiments, a first phase comprises at least one oligonucleotide and at least one penetration enhancer, while a second phase comprises at least one penetration enhancer. In some embodiments, a first phase comprises at least one oligonucleotide and at least one penetration enhancer, while a second phase comprises at least one penetration enhancer and substantially no oligonucleotide. In some embodiments, at least one phase is compounded with at least one degradation retardant, such as a coating or a matrix, which delays release of the contents of that phase. In some embodiments, at least one phase In some embodiments, a first phase comprises at least one oligonucleotide, at least one penetration enhancer, while a second phase comprises at least one penetration enhancer and a release-

retardant. In particular embodiments, an oral oligonucleotide comprises a first phase comprising particles containing an oligonucleotide and a penetration enhancer, and a second phase comprising particles coated with a release-retarding agent and containing penetration enhancer.

A variety of bile salts also function as penetration enhancers to facilitate the uptake and bioavailability of drugs. The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 *In: Goodman & Gilman=s The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (CDCA, sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Swinyard, Chapter 39 *In: Remington=s Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, *7*, 1; Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, 1992, *263*, 25; Yamashita *et al.*, *J. Pharm. Sci.*, 1990, *79*, 579).

In some embodiments, penetration enhancers useful in some embodiments of present invention are mixtures of penetration enhancing compounds. One such penetration enhancer is a mixture of UDCA (and/or CDCA) with capric and/or lauric acids or salts thereof e.g. sodium. Such mixtures are useful for enhancing the delivery of biologically active substances across mucosal membranes, in particular intestinal mucosa. Other penetration enhancer mixtures comprise about 5-95% of bile acid or salt(s) UDCA and/or CDCA with 5-95% capric and/or lauric acid. Particular penetration enhancers are mixtures of the sodium salts of UDCA, capric acid and lauric acid in a ratio of about 1:2:2 respectively. Anther such penetration enhancer is a mixture of capric and lauric acid (or salts thereof) in a 0.01:1 to 1:0.01 ratio (mole basis). In particular embodiments capric acid and lauric acid are present in molar ratios of e.g. about 0.1:1 to about 1:0.1, in particular about 0.5:1 to about 1:0.5.

Other excipients include chelating agents, i.e. compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the alimentary and other mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, 1993, *618*, 315). Chelating agents of the invention include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1; Buur *et al.*, *J. Control Rel.*, 1990, *14*, 43).

As used herein, non-chelating non-surfactant penetration enhancers may be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary and other mucosal membranes (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, *7*, 1). This class of penetration enhancers includes, but is not limited to, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, 1987, *39*, 621).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), can be used.

Some oral oligonucleotide compositions also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which may be inert (*i.e.*, does not possess biological activity *per se*) or may be necessary for transport, recognition or pathway activation or mediation, or is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic

acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, 1995, *5*, 115; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, 1996, *6*, 177).

A "pharmaceutical carrier" or "excipient" may be a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, EXPLOTAB); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

Oral oligonucleotide compositions may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present

invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention.

What is claimed is:

1. An antisense compound 8 to 50 nucleobases in length, wherein said compound specifically hybridizes with nucleotides 2920-3420 as set forth in SEQ ID NO:3 and inhibits expression of mRNA encoding human apolipoprotein B after 16 to 24 hours by at least 30% in 80% confluent HepG2 cells in culture at a concentration of 150 nM.

2. The antisense compound of claim 1, wherein said compound specifically hybridizes with nucleotides 3230-3288 as set forth in SEQ ID NO:3 and inhibits expression of mRNA encoding human apolipoprotein B after 16 to 24 hours by at least 30% in 80% confluent HepG2 cells in culture at a concentration of 150 nM.

3. The antisense compound of claim 2 that is an antisense oligonucleotide.

4. The antisense compound of claim 3, wherein the antisense oligonucleotide is an oligonucleotide mimetic compound.

5. The antisense compound of claim 2, twelve to thirty nucleobases in length.

6. The antisense compound of claim 5, fourteen to twenty nucleobases in length.

7. The antisense compound of claim 4, wherein the oligonucleotide mimetic compound comprises at least one phosphorothioate linkage.

8. The antisense compound of claim 4, wherein the oligonucleotide mimetic compound comprises at least one 2'-O-methoxyethyl sugar moiety.

9. The antisense compound of claim 4, wherein the oligonucleotide mimetic compound comprises at least one 5-methylcytosine.

10. The antisense compound of claim 2, wherein the antisense compound is a chimeric antisense compound.

11. The antisense compound of claim 10, wherein the chimeric antisense compound is a chimeric phosphorothioate antisense compound.

12. The antisense compound of claim 11, wherein the chimeric phosphorothioate antisense compound comprises 2'-methoxyethoxyl nucleotide wings and a 2'-deoxynucleotide gap.

13. The antisense compound of claim 12, wherein the chimeric phosporothioate antisense compound comprises ten 2'-deoxynucleotides.

14. The antisense compound of any one of claims 1-13, wherein said antisense compound inhibits expression of mRNA encoding human apolipoprotein B after 16 to 24 hours by at least 50% in 80% confluent HepG2 cells in culture at a concentration of 150 nM.

15. The antisense compound of any one of claims 1-13, wherein at least one nucleobase is covalently linked to a conjugate.

16. A composition comprising the antisense compound of any one of claims 1-13 and a pharmaceutically acceptable carrier or diluent.

17. The composition of claim 16 further comprising a colloidal dispersion system.

18. A composition comprising an antisense compound of any of claims 1-13 hybridized to a complementary strand.

19. The composition of claim 18, wherein the hybridization of the antisense compound to the complementary strand forms at least one blunt end.

20. The composition of claim 19, wherein the hybridization of the antisense compound to the complementary strand forms two blunt ends.

21. An antisense oligonucleotide compound 8 to 50 nucleobases in length comprising at least 8 contiguous nucleotides of SEQ ID NO:247.

22. The antisense oligonucleotide compound of claim 21, wherein the antisense oligonucleotide compound has a sequence comprising SEQ ID NO:247.

23. The antisense oligonucleotide compound of claim 22, twelve to thirty nucleobases in length.

24. The antisense oligonucleotide compound of claim 23, fourteen to twenty nucleobases in length.

25. The antisense oligonucleotide compound of claim 24, wherein the antisense oligonucleotide compound has a sequence consisting of SEQ ID NO:247.

26. The antisense oligonucleotide compound of claim 25, wherein the antisense oligonucleotide compound is an oligonucleotide mimetic compound.

27. The antisense oligonucleotide compound of claim 26, wherein the oligonucleotide mimetic compound is a chimeric phosporothioate oligonucleotide compound.

28. The antisense oligonucleotide compound of claim 27, wherein the chimeric phosporothioate oligonucleotide compound comprises 2'-methoxyethoxyl nucleotide wings and a 2'-deoxynucleotide gap.

29. The antisense oligonucleotide compound of claim 28, wherein the chimeric phosporothioate oligonucleotide compound comprises ten 2'-deoxynucleotides.

30. The antisense oligonucleotide compound of any one of claims 21-29, wherein at least one oligonucleotide is covalently linked to a conjugate.

31. A composition comprising the antisense oligonucleotide compound of any of claims 21-29 and a pharmaceutically acceptable carrier or diluent.

32. The composition of claim 31 further comprising a colloidal dispersion system.

33. A composition comprising an oligonucleotide compound of any of claims 22-29 hybridized to a complementary strand.

34. The composition of claim 33, wherein the hybridization of the oligonucleotide compound to the complementary strand forms at least one blunt end.

35. The composition of claim 34, wherein the hybridization of the oligonucleotide compound to the complementary strand forms two blunt ends.

36. A method of inhibiting the expression of apolipoprotein B in cells or tissues comprising contacting said cells or tissues with a compound of claim 2 under conditions such that expression of apolipoprotein B is inhibited.

37. A method of inhibiting the expression of apolipoprotein B in cells or tissues comprising contacting said cells or tissues with a compound of claim 21 under conditions such that expression of apolipoprotein B is inhibited.

38. The method of claim 36 or claim 37, wherein the cells or tissues are contacted *in vivo*.

39. The method of claim 38, wherein said contacting comprises the step of administering the compound to an animal.

40. The method of claim 39, wherein the animal is a human.

41. The method of claim 40, wherein the human has a disease or condition associated with apolipoprotein B expression and a therapeutically or prophylactically effective amount of the compound is administered.

42. The method of claim 41, wherein the human has a condition associated with abnormal lipid metabolism.

43. The method of claim 41, wherein the human has a condition associated with abnormal cholesterol metabolism.

44. The method of claim 41, wherein the human has a cardiovascular disease.

45. The method of claim 44, wherein the cardiovascular disease is atherosclerosis.

46. The method of claim 41, wherein the human has an abnormal metabolic condition associated with apolipoprotein B expression.

47. The method of claim 46, wherein the abnormal metabolic condition is hyperlipidemia.

48. The method of claim 41, wherein the human has diabetes.

49. The method of claim 41, wherein the human is obese.

50. The method of claim 40, wherein an effective amount of the compound is administered to prevent a disease or condition associated with apolipoprotein B expression.

51. The method of claim 40, wherein an effective amount of the compound is administered to delay a disease or condition associated with apolipoprotein B expression.

52. A method of preventing or delaying the onset of an increase in glucose levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1.

53. A method of preventing or delaying the onset of an increase in glucose levels in an animal comprising administering to said animal a therapeutically or

prophylactically effective amount of the compound of claim 22.

54. The method of claim 52 or claim 53 wherein the animal is a human.

55. The method of claim 54 wherein the glucose levels are serum or plasma glucose levels.

56. A method of modulating serum cholesterol levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 or 21.

57. The method of claim 56 wherein the animal is a human.

58. A method of modulating lipoprotein levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1.

59. A method of modulating lipoprotein levels in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 22.

60. The method of claim 58 or claim 59 wherein the animal is a human.

61. The method of claim 60 wherein the lipoprotein is VLDL.

62. The method of claim 60 wherein the lipoprotein is HDL.

63. The method of claim 60 wherein the lipoprotein is LDL.

64. The method of any one of claims 39, 52, 53, 56, 58, and 59 wherein the compound is administered intravenously.

65. The method of any one of claims 39, 52, 53, 56, 58, and 59 wherein the compound is administered subcutaneously.

66. An antisense oligonucleotide compound 20 nucleobases in length having a sequence of nucleobases as set forth in SEQ ID NO:247 and comprising 5-methylcytidine at nucleobases 2, 3, 5, 9, 12, 15, 17, 19, and 20, wherein every internucleoside linkage is a phosphothioate linkage, nucleobases 1-5 and 16-20 comprise a 2'-methoxyethoxyl modification, and nucleobases 6-15 are deoxynucleotides.

67. The antisense oligonucleotide compound of claim 66, wherein at least one oligonucleotide is covalently linked to a conjugate.

68. A composition comprising the antisense oligonucleotide compound of claim 66 and a pharmaceutically acceptable carrier or diluent.

69. The composition of claim 68 further comprising a colloidal dispersion system.

70. A composition comprising the antisense oligonucleotide compound of claim 66 hybridized to a complementary strand.

71. A method of inhibiting the expression of apolipoprotein B in cells or tissues comprising contacting said cells or tissues with a compound of

claim 66 so that expression of apolipoprotein B is inhibited.

72. The method of claim 71, wherein the cells or tissues are contacted *in vivo*.

73. The method of claim 72, wherein said contacting comprises the step of administering the compound to an animal.

74. The method of claim 73, wherein the animal is a human.

75. The method of claim 74, wherein the human has a disease or condition associated with apolipoprotein B expression and a therapeutically or prophylactically effective amount of the compound is administered.

76. The method of claim 75, wherein the human has a condition associated with abnormal lipid metabolism.

77. The method of claim 75, wherein the human has a condition associated with abnormal cholesterol metabolism.

78. The method of claim 75, wherein the human has a cardiovascular disease.

79. The method of claim 78, wherein the cardiovascular disease is atherosclerosis.

80. The method of claim 75, wherein the human has an abnormal metabolic condition associated with apolipoprotein B expression.

81. The method of claim 80, wherein the abnormal metabolic condition is hyperlipidemia.

82. The method of claim 75, wherein the human has diabetes.

83. The method of claim 75, wherein the human is obese.

84. The method of claim 74, wherein an effective amount of the compound is administered to prevent a disease or condition associated with apolipoprotein B expression.

85. The method of claim 74, wherein an effective amount of the compound is administered to delay a disease or condition associated with apolipoprotein B expression.

86. A method of preventing or delaying the onset of an increase in glucose levels in a human comprising administering to said human a therapeutically or prophylactically effective amount of the compound of claim 66.

87. The method of claim 86 wherein the glucose levels are serum glucose levels.

88. The method of claim 86 wherein the glucose levels are plasma glucose levels.

89. A method of modulating serum cholesterol levels in a human comprising administering to said human a therapeutically or prophylactically effective amount of the compound of claim 66.

90. A method of modulating lipoprotein levels in a human comprising administering to said human a therapeutically or prophylactically effective amount of the compound of claim 66.

91. The method of claim 90 wherein the lipoprotein is VLDL.

92. The method of claim 90 wherein the lipoprotein is HDL.

93. The method of claim 90 wherein the lipoprotein is LDL.

94. The method of any one of claims 73-93 wherein the compound is administered intravenously.

95. The method of any one of claims 73-93 wherein the compound is administered subcutaneously.

96. The method of any one of claims 73-93 wherein the compound is administered orally.

97. A kit comprising a compound of any one of claims 1-15, 21-30, and 66-67.

98. A compound comprising a first nucleobase strand hybridized to a second nucleobase strand, each strand 8 to 50 nucleobases in length, said first nucleobase strand comprising a sequence of at least 8 contiguous nucleobases of the sequence set forth in SEQ ID NO:3, said second nucleobase strand comprising a sequence sufficiently complementary to said first strand so as to permit stable hybridization, said compound inhibiting expression of mRNA encoding human apolipoprotein B after 16 to 24 hours by at least 30% in 80% confluent HepG2 cells in culture at a concentration of 100 nM.

99. The compound of claim 98, wherein the first strand comprises a sequence of 12 to 30 contiguous nucleobases of the sequence set forth in SEQ ID NO:3.

100. The compound of claim 98, wherein the first strand comprises a sequence of 20 contiguous nucleobases of the sequence set forth in SEQ ID NO:3.

101. The compound of claims 98, 99, or 100, wherein the second strand comprises a sequence perfectly complimentary to at least 8 contiguous nucleobases of the sequence set forth in SEQ ID NO:3.

102. The compound of claim 101, wherein the second strand comprises a sequence perfectly complimentary to 12 to 30 nucleobases of the sequence set forth in SEQ ID NO:3.

103. The compound of claim 101, wherein the second strand comprises a sequence perfectly complimentary to 20 nucleobases of the sequence set forth in SEQ ID NO:3.

104. The compound of any of claims 98-103, wherein at least one strand comprises RNA.

105. The compound of any of claims 98-104, wherein at least one strand comprises one or more deoxynucleosides.

106. The compound of any of claims 98-105, wherein the hybridized strands form at least one overhanging end.

107. The compound of claim 106, wherein the overhanging end comprises at least one modified base.

108. The compound of any of claims 98-107, wherein said compound inhibits expression of mRNA encoding human apolipoprotein B after 16 to 24 hours by at least 50% in 80% confluent HepG2 cells in culture at a concentration of 100 nM.

109. A vesicle comprising a compound any of claims 98-108.

110. The vesicle of claim 109, wherein the vesicle is a liposome.

111. A composition comprising the compound of any one of claims 98-108 and a pharmaceutically acceptable carrier or diluent.

112. The composition of claim 111 further comprising a colloidal dispersion system.

113. A method of inhibiting the expression of apolipoprotein B in cells or tissues comprising contacting said cells or tissues with the compound of any one of claims 98-108 under conditions such that expression of apolipoprotein B is inhibited.

114. The method of claim 113, wherein the cells or tissues are contacted *in vivo*.

115. The method of claim 114, wherein said contacting comprises the step of administering the compound to an animal.

116. The method of claim 115, wherein the animal is a human.

117. The method of claim 116, wherein the human has a condition associated with apolipoprotein B expression and a therapeutically or prophylactically effective amount of the compound is administered.

118. The method of claim 117, wherein said condition is associated with abnormal lipid metabolism.

119. The method of claim 117, wherein said condition is associated with abnormal cholesterol metabolism.

120. The method of claim 117, wherein said condition is cardiovascular disease.

121. The method of claim 120, wherein the cardiovascular disease is atherosclerosis.

122. The method of claim 117, wherein said condition is an abnormal metabolic condition associated with apolipoprotein B expression.

123. The method of claim 122, wherein the abnormal metabolic condition associated with apolipoprotein B expression is hyperlipidemia.

124. The method of claim 117, wherein the condition is diabetes.

125. The method of claim 117, wherein the condition is obesity.

126. The method of claim 116, wherein an effective amount of the compound is administered to prevent a condition associated with apolipoprotein B expression.

127. The method of claim 126, wherein an effective amount of the compound is administered to delay a condition associated with apolipoprotein B expression.

128. A method of reducing lipoprotein(a) secretion by hepatocytes comprising:

(a) contacting hepatocytes with an amount of a composition comprising a non-catalytic compound 8 to 50 nucleobases in length that specifically hybridizes

with mRNA encoding human apolipoprotein B and inhibits expression of the mRNA after 16 to 24 hours by at least 30% in 80% confluent HepG2 cells in culture at a concentration of 150 nM, wherein said amount is effective to inhibit expression of apolipoprotein B in the hepatocytes; and

(b) measuring lipoprotein(a) secretion by the hepatocytes.

129. The method of claim 128, wherein the non-catalytic compound specifically hybridizes with nucleotides 3230-3288 as set forth in SEQ ID NO:3.

130. The method of claim 129, wherein the non-catalytic compound comprises a sequence of nucleobases as set forth in SEQ ID NO:247.

131. The method of any of claims 128-130, wherein the non-catalytic compound is a antisense olionucleotide mimetic.

132. A method of a treating a condition associated with apolipoprotein B expression in a primate comprising administering to the primate a therapeutically or prophylactically effective amount of a non-catalytic compound 8 to 50 nucleobases in length that specifically hybridizes with mRNA encoding human apolipoprotein B and inhibits expression of the mRNA after 16 to 24 hours by at least 30% in 80% confluent HepG2 cells in culture at a concentration of 150 nM.

133. The method of claim 132, wherein the primate is a human.

134. The method of claim 133 wherein the condition is selected from the group consisting of abnormal lipid metabolism, abnormal cholesterol metabolism, cardiovascular disease, hyperlipidemia, diabetes, and obesity.

135. A method of reducing apolipoprotein B expression in the liver of an animal, comprising administering to the animal between 2 mg/kg and 20 mg/kg of a non-catalytic compound 8 to 50 nucleobases in length that specifically hybridizes with mRNA encoding human apolipoprotein B by at least 30% in 80% confluent HepG2 cells in culture at a concentration of 150 nM.

136. The method of claim 135, wherein the compound is administered subcutaneously.

137. The method of claim 135, wherein the compound is administered intraveneously.

138. The method of claim 135, wherein the compound is administered orally.

139. The method of any one of claims 135-138, wherein administration to the animal is repeated.

140. The method of any one of claims 135-139, wherein the animal is a human.

141. Use of a compound of any one of claims 1-15, 21-30, 66-67, and 98-108 in the production of a medicament.

142. Use of a compound of any one of claims 1-15, 21-30, 66-67, and 98-108 in a medicament for altering lipid metabolism.

143. Use of a compound of any one of claims 1-15, 21-30, 66-67, and 98-108 in a medicament for a disease or condition associated with apolipoprotein B expression.

144. A method of making a compound of any one of claims 98-108 comprising specifically hybridizing *in vitro* a first nucleobase strand comprising a sequence of at least 8 contiguous nucleobases of the sequence set forth in SEQ ID NO:3 to a second nucleobase strand comprising a sequence sufficiently complementary to said first strand so as to permit stable hybridization.

SEQUENCE LISTING

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<120> ANTISENSE MODULATION OF APOLIPOPROTEIN B EXPRESSION

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         Met Asp Pro Pro Arg Pro Ala Leu Leu Ala Leu Leu Ala Leu
         1               5                   10

cct gcg ctg ctg ctg ctg ctg ctg gcg ggc gcc agg gcc gaa gag gaa      218
Pro Ala Leu Leu Leu Leu Leu Leu Ala Gly Ala Arg Ala Glu Glu Glu
15                  20                  25                  30

atg ctg gaa aat gtc agc ctg gtc tgt cca aaa gat gcg acc cga ttc      266
Met Leu Glu Asn Val Ser Leu Val Cys Pro Lys Asp Ala Thr Arg Phe
                35                  40                  45
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aag cac ctc cgg aag tac aca tac aac tat gag gct gag agt tcc agt    314
Lys His Leu Arg Lys Tyr Thr Tyr Asn Tyr Glu Ala Glu Ser Ser Ser
            50                  55                  60

gga gtc cct ggg act gct gat tca aga agt gcc acc agg atc aac tgc    362
Gly Val Pro Gly Thr Ala Asp Ser Arg Ser Ala Thr Arg Ile Asn Cys
        65                  70                  75

aag gtt gag ctg gag gtt ccc cag ctc tgc agc ttc atc ctg aag acc    410
Lys Val Glu Leu Glu Val Pro Gln Leu Cys Ser Phe Ile Leu Lys Thr
    80                  85                  90

agc cag tgc acc ctg aaa gag gtg tat ggc ttc aac cct gag ggc aaa    458
Ser Gln Cys Thr Leu Lys Glu Val Tyr Gly Phe Asn Pro Glu Gly Lys
95                  100                 105                 110

gcc ttg ctg aag aaa acc aag aac tct gag gag ttt gct gca gcc atg    506
Ala Leu Leu Lys Lys Thr Lys Asn Ser Glu Glu Phe Ala Ala Ala Met
                115                 120                 125

tcc agg tat gag ctc aag ctg gcc att cca gaa ggg aag cag gtt ttc    554
Ser Arg Tyr Glu Leu Lys Leu Ala Ile Pro Glu Gly Lys Gln Val Phe
            130                 135                 140

ctt tac ccg gag aaa gat gaa cct act tac atc ctg aac atc aag agg    602
Leu Tyr Pro Glu Lys Asp Glu Pro Thr Tyr Ile Leu Asn Ile Lys Arg
        145                 150                 155

ggc atc att tct gcc ctc ctg gtt ccc cca gag aca gaa gaa gcc aag    650
Gly Ile Ile Ser Ala Leu Leu Val Pro Pro Glu Thr Glu Glu Ala Lys
    160                 165                 170

caa gtg ttg ttt ctg gat acc gtg tat gga aac tgc tcc act cac ttt    698
Gln Val Leu Phe Leu Asp Thr Val Tyr Gly Asn Cys Ser Thr His Phe
175                 180                 185                 190

acc gtc aag acg agg aag ggc aat gtg gca aca gaa ata tcc act gaa    746
Thr Val Lys Thr Arg Lys Gly Asn Val Ala Thr Glu Ile Ser Thr Glu
                195                 200                 205

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Arg Asp Leu Gly Gln Cys Asp Arg Phe Lys Pro Ile Arg Thr Gly Ile
            210                 215                 220

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Ser Pro Leu Ala Leu Ile Lys Gly Met Thr Arg Pro Leu Ser Thr Leu
        225                 230                 235

atc agc agc agc cag tcc tgt cag tac aca ctg gac gct aag agg aag    890
Ile Ser Ser Ser Gln Ser Cys Gln Tyr Thr Leu Asp Ala Lys Arg Lys
    240                 245                 250

cat gtg gca gaa gcc atc tgc aag gag caa cac ctc ttc ctg cct ttc    938
His Val Ala Glu Ala Ile Cys Lys Glu Gln His Leu Phe Leu Pro Phe
255                 260                 265                 270

tcc tac aac aat aag tat ggg atg gta gca caa gtg aca cag act ttg    986
Ser Tyr Asn Asn Lys Tyr Gly Met Val Ala Gln Val Thr Gln Thr Leu
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Lys Leu Glu Asp Thr Pro Lys Ile Asn Ser Arg Phe Phe Gly Glu Gly
            290                 295                 300

act aag aag atg ggc ctc gca ttt gag agc acc aaa tcc aca tca cct    1082
Thr Lys Lys Met Gly Leu Ala Phe Glu Ser Thr Lys Ser Thr Ser Pro
        305                 310                 315

cca aag cag gcc gaa gct gtt ttg aag act ctc cag gaa ctg aaa aaa    1130
Pro Lys Gln Ala Glu Ala Val Leu Lys Thr Leu Gln Glu Leu Lys Lys
    320                 325                 330

cta acc atc tct gag caa aat atc cag aga gct aat ctc ttc aat aag    1178
Leu Thr Ile Ser Glu Gln Asn Ile Gln Arg Ala Asn Leu Phe Asn Lys
335                 340                 345                 350

ctg gtt act gag ctg aga ggc ctc agt gat gaa gca gtc aca tct ctc    1226
Leu Val Thr Glu Leu Arg Gly Leu Ser Asp Glu Ala Val Thr Ser Leu
                355                 360                 365

ttg cca cag ctg att gag gtg tcc agc ccc atc act tta caa gcc ttg    1274
Leu Pro Gln Leu Ile Glu Val Ser Ser Pro Ile Thr Leu Gln Ala Leu
            370                 375                 380

gtt cag tgt gga cag cct cag tgc tcc act cac atc ctc cag tgg ctg    1322
Val Gln Cys Gly Gln Pro Gln Cys Ser Thr His Ile Leu Gln Trp Leu
        385                 390                 395

aaa cgt gtg cat gcc aac ccc ctt ctg ata gat gtg gtc acc tac ctg    1370
Lys Arg Val His Ala Asn Pro Leu Leu Ile Asp Val Val Thr Tyr Leu
    400                 405                 410

gtg gcc ctg atc ccc gag ccc tca gca cag cag ctg cga gag atc ttc    1418
Val Ala Leu Ile Pro Glu Pro Ser Ala Gln Gln Leu Arg Glu Ile Phe
415                 420                 425                 430

aac atg gcg agg gat cag cgc agc cga gcc acc ttg tat gcg ctg agc    1466
Asn Met Ala Arg Asp Gln Arg Ser Arg Ala Thr Leu Tyr Ala Leu Ser
                435                 440                 445

cac gcg gtc aac aac tat cat aag aca aac cct aca ggg acc cag gag    1514
His Ala Val Asn Asn Tyr His Lys Thr Asn Pro Thr Gly Thr Gln Glu
            450                 455                 460

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Leu Leu Asp Ile Ala Asn Tyr Leu Met Glu Gln Ile Gln Asp Asp Cys
        465                 470                 475

act ggg gat gaa gat tac acc tat ttg att ctg cgg gtc att gga aat    1610
Thr Gly Asp Glu Asp Tyr Thr Tyr Leu Ile Leu Arg Val Ile Gly Asn
    480                 485                 490

atg ggc caa acc atg gag cag tta act cca gaa ctc aag tct tca atc    1658
Met Gly Gln Thr Met Glu Gln Leu Thr Pro Glu Leu Lys Ser Ser Ile
495                 500                 505                 510

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Leu Lys Cys Val Gln Ser Thr Lys Pro Ser Leu Met Ile Gln Lys Ala
                515                 520                 525

gcc atc cag gct ctg cgg aaa atg gag cct aaa gac aag gac cag gag    1754
Ala Ile Gln Ala Leu Arg Lys Met Glu Pro Lys Asp Lys Asp Gln Glu
            530                 535                 540
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gtt ctt ctt cag act ttc ctt gat gat gct tct ccg gga gat aag cga    1802
Val Leu Leu Gln Thr Phe Leu Asp Asp Ala Ser Pro Gly Asp Lys Arg
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ctg gct gcc tat ctt atg ttg atg agg agt cct tca cag gca gat att    1850
Leu Ala Ala Tyr Leu Met Leu Met Arg Ser Pro Ser Gln Ala Asp Ile
    560                 565                 570

aac aaa att gtc caa att cta cca tgg gaa cag aat gag caa gtg aag    1898
Asn Lys Ile Val Gln Ile Leu Pro Trp Glu Gln Asn Glu Gln Val Lys
575                 580                 585                 590

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gat atc caa gat ctg aaa aag tta gtg aaa gaa gct ctg aaa gaa tct    1994
Asp Ile Gln Asp Leu Lys Lys Leu Val Lys Glu Ala Leu Lys Glu Ser
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Gln Leu Pro Thr Val Met Asp Phe Arg Lys Phe Ser Arg Asn Tyr Gln
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Ile Glu Gly Asn Leu Ile Phe Asp Pro Asn Asn Tyr Leu Pro Lys Glu
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Ser Met Leu Lys Thr Thr Leu Thr Ala Phe Gly Phe Ala Ser Ala Asp
                675                 680                 685

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Leu Ile Glu Ile Gly Leu Glu Gly Lys Gly Phe Glu Pro Thr Leu Glu
            690                 695                 700

gct ctt ttt ggg aag caa gga ttt ttc cca gac agt gtc aac aaa gct    2282
Ala Leu Phe Gly Lys Gln Gly Phe Phe Pro Asp Ser Val Asn Lys Ala
        705                 710                 715

ttg tac tgg gtt aat ggt caa gtt cct gat ggt gtc tct aag gtc tta    2330
Leu Tyr Trp Val Asn Gly Gln Val Pro Asp Gly Val Ser Lys Val Leu
    720                 725                 730

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Val Asp His Phe Gly Tyr Thr Lys Asp Asp Lys His Glu Gln Asp Met
735                 740                 745                 750

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Val Asn Gly Ile Met Leu Ser Val Glu Lys Leu Ile Lys Asp Leu Lys
                755                 760                 765

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Ser Lys Glu Val Pro Glu Ala Arg Ala Tyr Leu Arg Ile Leu Gly Glu
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    800                 805                 810

gag gtc atc agg aag ggc tca aag aat gac ttt ttt ctt cac tac atc    2618
Glu Val Ile Arg Lys Gly Ser Lys Asn Asp Phe Phe Leu His Tyr Ile
815                 820                 825                 830

ttc atg gag aat gcc ttt gaa ctc ccc act gga gct gga tta cag ttg    2666
Phe Met Glu Asn Ala Phe Glu Leu Pro Thr Gly Ala Gly Leu Gln Leu
                835                 840                 845

caa ata tct tca tct gga gtc att gct ccc gga gcc aag gct gga gta    2714
Gln Ile Ser Ser Ser Gly Val Ile Ala Pro Gly Ala Lys Ala Gly Val
            850                 855                 860

aaa ctg gaa gta gcc aac atg cag gct gaa ctg gtg gca aaa ccc tcc    2762
Lys Leu Glu Val Ala Asn Met Gln Ala Glu Leu Val Ala Lys Pro Ser
        865                 870                 875

gtg tct gtg gag ttt gtg aca aat atg ggc atc atc att ccg gac ttc    2810
Val Ser Val Glu Phe Val Thr Asn Met Gly Ile Ile Ile Pro Asp Phe
    880                 885                 890

gct agg agt ggg gtc cag atg aac acc aac ttc ttc cac gag tcg ggt    2858
Ala Arg Ser Gly Val Gln Met Asn Thr Asn Phe Phe His Glu Ser Gly
895                 900                 905                 910

ctg gag gct cat gtt gcc cta aaa gct ggg aag ctg aag ttt atc att    2906
Leu Glu Ala His Val Ala Leu Lys Ala Gly Lys Leu Lys Phe Ile Ile
                915                 920                 925

cct tcc cca aag aga cca gtc aag ctg ctc agt gga ggc aac aca tta    2954
Pro Ser Pro Lys Arg Pro Val Lys Leu Leu Ser Gly Gly Asn Thr Leu
            930                 935                 940

cat ttg gtc tct acc acc aaa acg gag gtg atc cca cct ctc att gag    3002
His Leu Val Ser Thr Thr Lys Thr Glu Val Ile Pro Pro Leu Ile Glu
        945                 950                 955

aac agg cag tcc tgg tca gtt tgc aag caa gtc ttt cct ggc ctg aat    3050
Asn Arg Gln Ser Trp Ser Val Cys Lys Gln Val Phe Pro Gly Leu Asn
    960                 965                 970

tac tgc acc tca ggc gct tac tcc aac gcc agc tcc aca gac tcc gcc    3098
Tyr Cys Thr Ser Gly Ala Tyr Ser Asn Ala Ser Ser Thr Asp Ser Ala
975                 980                 985                 990

tcc tac tat ccg ctg acc ggg gac acc aga tta gag ctg gaa ctg agg    3146
Ser Tyr Tyr Pro Leu Thr Gly Asp Thr Arg Leu Glu Leu Glu Leu Arg
                995                 1000                1005

cct aca gga gag att gag cag tat tct gtc agc gca acc tat gag ctc    3194
Pro Thr Gly Glu Ile Glu Gln Tyr Ser Val Ser Ala Thr Tyr Glu Leu
            1010                1015                1020

cag aga gag gac aga gcc ttg gtg gat acc ctg aag ttt gta act caa    3242
Gln Arg Glu Asp Arg Ala Leu Val Asp Thr Leu Lys Phe Val Thr Gln
        1025                1030                1035

gca gaa ggt gcg aag cag act gag gct acc atg aca ttc aaa tat aat    3290
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Ala Glu Gly Ala Lys Gln Thr Glu Ala Thr Met Thr Phe Lys Tyr Asn
    1040                1045                1050

cgg cag agt atg acc ttg tcc agt gaa gtc caa att ccg gat ttt gat    3338
Arg Gln Ser Met Thr Leu Ser Ser Glu Val Gln Ile Pro Asp Phe Asp
1055                1060                1065                1070

gtt gac ctc gga aca atc ctc aga gtt aat gat gaa tct act gag ggc    3386
Val Asp Leu Gly Thr Ile Leu Arg Val Asn Asp Glu Ser Thr Glu Gly
                1075                1080                1085

aaa acg tct tac aga ctc acc ctg gac att cag aac aag aaa att act    3434
Lys Thr Ser Tyr Arg Leu Thr Leu Asp Ile Gln Asn Lys Lys Ile Thr
            1090                1095                1100

gag gtc gcc ctc atg ggc cac cta agt tgt gac aca aag gaa gaa aga    3482
Glu Val Ala Leu Met Gly His Leu Ser Cys Asp Thr Lys Glu Glu Arg
        1105                1110                1115

aaa atc aag ggt gtt att tcc ata ccc cgt ttg caa gca gaa gcc aga    3530
Lys Ile Lys Gly Val Ile Ser Ile Pro Arg Leu Gln Ala Glu Ala Arg
    1120                1125                1130

agt gag atc ctc gcc cac tgg tcg cct gcc aaa ctg ctt ctc caa atg    3578
Ser Glu Ile Leu Ala His Trp Ser Pro Ala Lys Leu Leu Leu Gln Met
1135                1140                1145                1150

gac tca tct gct aca gct tat ggc tcc aca gtt tcc aag agg gtg gca    3626
Asp Ser Ser Ala Thr Ala Tyr Gly Ser Thr Val Ser Lys Arg Val Ala
                1155                1160                1165

tgg cat tat gat gaa gag aag att gaa ttt gaa tgg aac aca ggc acc    3674
Trp His Tyr Asp Glu Glu Lys Ile Glu Phe Glu Trp Asn Thr Gly Thr
            1170                1175                1180

aat gta gat acc aaa aaa atg act tcc aat ttc cct gtg gat ctc tcc    3722
Asn Val Asp Thr Lys Lys Met Thr Ser Asn Phe Pro Val Asp Leu Ser
        1185                1190                1195

gat tat cct aag agc ttg cat atg tat gct aat aga ctc ctg gat cac    3770
Asp Tyr Pro Lys Ser Leu His Met Tyr Ala Asn Arg Leu Leu Asp His
    1200                1205                1210

aga gtc cct gaa aca gac atg act ttc cgg cac gtg ggt tcc aaa tta    3818
Arg Val Pro Glu Thr Asp Met Thr Phe Arg His Val Gly Ser Lys Leu
1215                1220                1225                1230

ata gtt gca atg agc tca tgg ctt cag aag gca tct ggg agt ctt cct    3866
Ile Val Ala Met Ser Ser Trp Leu Gln Lys Ala Ser Gly Ser Leu Pro
                1235                1240                1245

tat acc cag act ttg caa gac cac ctc aat agc ctg aag gag ttc aac    3914
Tyr Thr Gln Thr Leu Gln Asp His Leu Asn Ser Leu Lys Glu Phe Asn
            1250                1255                1260

ctc cag aac atg gga ttg cca gac ttc cac atc cca gaa aac ctc ttc    3962
Leu Gln Asn Met Gly Leu Pro Asp Phe His Ile Pro Glu Asn Leu Phe
        1265                1270                1275

tta aaa agc gat ggc cgg gtc aaa tat acc ttg aac aag aac agt ttg    4010
Leu Lys Ser Asp Gly Arg Val Lys Tyr Thr Leu Asn Lys Asn Ser Leu
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1280 1285 1290

aaa att gag att cct ttg cct ttt ggt ggc aaa tcc tcc aga gat cta 4058
Lys Ile Glu Ile Pro Leu Pro Phe Gly Gly Lys Ser Ser Arg Asp Leu
1295 1300 1305 1310

aag atg tta gag act gtt agg aca cca gcc ctc cac ttc aag tct gtg 4106
Lys Met Leu Glu Thr Val Arg Thr Pro Ala Leu His Phe Lys Ser Val
1315 1320 1325

gga ttc cat ctg cca tct cga gag ttc caa gtc cct act ttt acc att 4154
Gly Phe His Leu Pro Ser Arg Glu Phe Gln Val Pro Thr Phe Thr Ile
1330 1335 1340

ccc aag ttg tat caa ctg caa gtg cct ctc ctg ggt gtt cta gac ctc 4202
Pro Lys Leu Tyr Gln Leu Gln Val Pro Leu Leu Gly Val Leu Asp Leu
1345 1350 1355

tcc acg aat gtc tac agc aac ttg tac aac tgg tcc gcc tcc tac agt 4250
Ser Thr Asn Val Tyr Ser Asn Leu Tyr Asn Trp Ser Ala Ser Tyr Ser
1360 1365 1370

ggt ggc aac acc agc aca gac cat ttc agc ctt cgg gct cgt tac cac 4298
Gly Gly Asn Thr Ser Thr Asp His Phe Ser Leu Arg Ala Arg Tyr His
1375 1380 1385 1390

atg aag gct gac tct gtg gtt gac ctg ctt tcc tac aat gtg caa gga 4346
Met Lys Ala Asp Ser Val Val Asp Leu Leu Ser Tyr Asn Val Gln Gly
1395 1400 1405

tct gga gaa aca aca tat gac cac aag aat acg ttc aca cta tca tgt 4394
Ser Gly Glu Thr Thr Tyr Asp His Lys Asn Thr Phe Thr Leu Ser Cys
1410 1415 1420

gat ggg tct cta cgc cac aaa ttt cta gat tcg aat atc aaa ttc agt 4442
Asp Gly Ser Leu Arg His Lys Phe Leu Asp Ser Asn Ile Lys Phe Ser
1425 1430 1435

cat gta gaa aaa ctt gga aac aac cca gtc tca aaa ggt tta cta ata 4490
His Val Glu Lys Leu Gly Asn Asn Pro Val Ser Lys Gly Leu Leu Ile
1440 1445 1450

ttc gat gca tct agt tcc tgg gga cca cag atg tct gct tca gtt cat 4538
Phe Asp Ala Ser Ser Ser Trp Gly Pro Gln Met Ser Ala Ser Val His
1455 1460 1465 1470

ttg gac tcc aaa aag aaa cag cat ttg ttt gtc aaa gaa gtc aag att 4586
Leu Asp Ser Lys Lys Lys Gln His Leu Phe Val Lys Glu Val Lys Ile
1475 1480 1485

gat ggg cag ttc aga gtc tct tcg ttc tat gct aaa ggc aca tat ggc 4634
Asp Gly Gln Phe Arg Val Ser Ser Phe Tyr Ala Lys Gly Thr Tyr Gly
1490 1495 1500

ctg tct tgt cag agg gat cct aac act ggc cgg ctc aat gga gag tcc 4682
Leu Ser Cys Gln Arg Asp Pro Asn Thr Gly Arg Leu Asn Gly Glu Ser
1505 1510 1515

aac ctg agg ttt aac tcc tcc tac ctc caa ggc acc aac cag ata aca 4730
Asn Leu Arg Phe Asn Ser Ser Tyr Leu Gln Gly Thr Asn Gln Ile Thr
1520 1525 1530

gga aga tat gaa gat gga acc ctc tcc ctc acc tcc acc tct gat ctg 4778
Gly Arg Tyr Glu Asp Gly Thr Leu Ser Leu Thr Ser Thr Ser Asp Leu
1535 1540 1545 1550

caa agt ggc atc att aaa aat act gct tcc cta aag tat gag aac tac 4826
Gln Ser Gly Ile Ile Lys Asn Thr Ala Ser Leu Lys Tyr Glu Asn Tyr
1555 1560 1565

gag ctg act tta aaa tct gac acc aat ggg aag tat aag aac ttt gcc 4874
Glu Leu Thr Leu Lys Ser Asp Thr Asn Gly Lys Tyr Lys Asn Phe Ala
1570 1575 1580

act tct aac aag atg gat atg acc ttc tct aag caa aat gca ctg ctg 4922
Thr Ser Asn Lys Met Asp Met Thr Phe Ser Lys Gln Asn Ala Leu Leu
1585 1590 1595

cgt tct gaa tat cag gct gat tac gag tca ttg agg ttc ttc agc ctg 4970
Arg Ser Glu Tyr Gln Ala Asp Tyr Glu Ser Leu Arg Phe Phe Ser Leu
1600 1605 1610

ctt tct gga tca cta aat tcc cat ggt ctt gag tta aat gct gac atc 5018
Leu Ser Gly Ser Leu Asn Ser His Gly Leu Glu Leu Asn Ala Asp Ile
1615 1620 1625 1630

tta ggc act gac aaa att aat agt ggt gct cac aag gcg aca cta agg 5066
Leu Gly Thr Asp Lys Ile Asn Ser Gly Ala His Lys Ala Thr Leu Arg
1635 1640 1645

att ggc caa gat gga ata tct acc agt gca acg acc aac ttg aag tgt 5114
Ile Gly Gln Asp Gly Ile Ser Thr Ser Ala Thr Thr Asn Leu Lys Cys
1650 1655 1660

agt ctc ctg gtg ctg gag aat gag ctg aat gca gag ctt ggc ctc tct 5162
Ser Leu Leu Val Leu Glu Asn Glu Leu Asn Ala Glu Leu Gly Leu Ser
1665 1670 1675

ggg gca tct atg aaa tta aca aca aat ggc cgc ttc agg gaa cac aat 5210
Gly Ala Ser Met Lys Leu Thr Thr Asn Gly Arg Phe Arg Glu His Asn
1680 1685 1690

gca aaa ttc agt ctg gat ggg aaa gcc gcc ctc aca gag cta tca ctg 5258
Ala Lys Phe Ser Leu Asp Gly Lys Ala Ala Leu Thr Glu Leu Ser Leu
1695 1700 1705 1710

gga agt gct tat cag gcc atg att ctg ggt gtc gac agc aaa aac att 5306
Gly Ser Ala Tyr Gln Ala Met Ile Leu Gly Val Asp Ser Lys Asn Ile
1715 1720 1725

ttc aac ttc aag gtc agt caa gaa gga ctt aag ctc tca aat gac atg 5354
Phe Asn Phe Lys Val Ser Gln Glu Gly Leu Lys Leu Ser Asn Asp Met
1730 1735 1740

atg ggc tca tat gct gaa atg aaa ttt gac cac aca aac agt ctg aac 5402
Met Gly Ser Tyr Ala Glu Met Lys Phe Asp His Thr Asn Ser Leu Asn
1745 1750 1755

att gca ggc tta tca ctg gac ttc tct tca aaa ctt gac aac att tac 5450
Ile Ala Gly Leu Ser Leu Asp Phe Ser Ser Lys Leu Asp Asn Ile Tyr
1760 1765 1770

agc tct gac aag ttt tat aag caa act gtt aat tta cag cta cag ccc 5498
Ser Ser Asp Lys Phe Tyr Lys Gln Thr Val Asn Leu Gln Leu Gln Pro

1775 1780 1785 1790

tat tct ctg gta act act tta aac agt gac ctg aaa tac aat gct ctg 5546
Tyr Ser Leu Val Thr Thr Leu Asn Ser Asp Leu Lys Tyr Asn Ala Leu
1795 1800 1805

gat ctc acc aac aat ggg aaa cta cgg cta gaa ccc ctg aag ctg cat 5594
Asp Leu Thr Asn Asn Gly Lys Leu Arg Leu Glu Pro Leu Lys Leu His
1810 1815 1820

gtg gct ggt aac cta aaa gga gcc tac caa aat aat gaa ata aaa cac 5642
Val Ala Gly Asn Leu Lys Gly Ala Tyr Gln Asn Asn Glu Ile Lys His
1825 1830 1835

atc tat gcc atc tct tct gct gcc tta tca gca agc tat aaa gca gac 5690
Ile Tyr Ala Ile Ser Ser Ala Ala Leu Ser Ala Ser Tyr Lys Ala Asp
1840 1845 1850

act gtt gct aag gtt cag ggt gtg gag ttt agc cat cgg ctc aac aca 5738
Thr Val Ala Lys Val Gln Gly Val Glu Phe Ser His Arg Leu Asn Thr
1855 1860 1865 1870

gac atc gct ggg ctg gct tca gcc att gac atg agc aca aac tat aat 5786
Asp Ile Ala Gly Leu Ala Ser Ala Ile Asp Met Ser Thr Asn Tyr Asn
1875 1880 1885

tca gac tca ctg cat ttc agc aat gtc ttc cgt tct gta atg gcc ccg 5834
Ser Asp Ser Leu His Phe Ser Asn Val Phe Arg Ser Val Met Ala Pro
1890 1895 1900

ttt acc atg acc atc gat gca cat aca aat ggc aat ggg aaa ctc gct 5882
Phe Thr Met Thr Ile Asp Ala His Thr Asn Gly Asn Gly Lys Leu Ala
1905 1910 1915

ctc tgg gga gaa cat act ggg cag ctg tat agc aaa ttc ctg ttg aaa 5930
Leu Trp Gly Glu His Thr Gly Gln Leu Tyr Ser Lys Phe Leu Leu Lys
1920 1925 1930

gca gaa cct ctg gca ttt act ttc tct cat gat tac aaa ggc tcc aca 5978
Ala Glu Pro Leu Ala Phe Thr Phe Ser His Asp Tyr Lys Gly Ser Thr
1935 1940 1945 1950

agt cat cat ctc gtg tct agg aaa agc atc agt gca gct ctt gaa cac 6026
Ser His His Leu Val Ser Arg Lys Ser Ile Ser Ala Ala Leu Glu His
1955 1960 1965

aaa gtc agt gcc ctg ctt act cca gct gag cag aca ggc acc tgg aaa 6074
Lys Val Ser Ala Leu Leu Thr Pro Ala Glu Gln Thr Gly Thr Trp Lys
1970 1975 1980

ctc aag acc caa ttt aac aac aat gaa tac agc cag gac ttg gat gct 6122
Leu Lys Thr Gln Phe Asn Asn Asn Glu Tyr Ser Gln Asp Leu Asp Ala
1985 1990 1995

tac aac act aaa gat aaa att ggc gtg gag ctt act gga cga act ctg 6170
Tyr Asn Thr Lys Asp Lys Ile Gly Val Glu Leu Thr Gly Arg Thr Leu
2000 2005 2010

gct gac cta act cta cta gac tcc cca att aaa gtg cca ctt tta ctc 6218
Ala Asp Leu Thr Leu Leu Asp Ser Pro Ile Lys Val Pro Leu Leu Leu
2015 2020 2025 2030

agt gag ccc atc aat atc att gat gct tta gag atg aga gat gcc gtt 6266

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Ser Glu Pro Ile Asn Ile Ile Asp Ala Leu Glu Met Arg Asp Ala Val
                2035                2040                2045

gag aag ccc caa gaa ttt aca att gtt gct ttt gta aag tat gat aaa      6314
Glu Lys Pro Gln Glu Phe Thr Ile Val Ala Phe Val Lys Tyr Asp Lys
            2050                2055                2060

aac caa gat gtt cac tcc att aac ctc cca ttt ttt gag acc ttg caa      6362
Asn Gln Asp Val His Ser Ile Asn Leu Pro Phe Phe Glu Thr Leu Gln
        2065                2070                2075

gaa tat ttt gag agg aat cga caa acc att ata gtt gta gtg gaa aac      6410
Glu Tyr Phe Glu Arg Asn Arg Gln Thr Ile Ile Val Val Val Glu Asn
    2080                2085                2090

gta cag aga aac ctg aag cac atc aat att gat caa ttt gta aga aaa      6458
Val Gln Arg Asn Leu Lys His Ile Asn Ile Asp Gln Phe Val Arg Lys
2095                2100                2105                2110

tac aga gca gcc ctg gga aaa ctc cca cag caa gct aat gat tat ctg      6506
Tyr Arg Ala Ala Leu Gly Lys Leu Pro Gln Gln Ala Asn Asp Tyr Leu
                2115                2120                2125

aat tca ttc aat tgg gag aga caa gtt tca cat gcc aag gag aaa ctg      6554
Asn Ser Phe Asn Trp Glu Arg Gln Val Ser His Ala Lys Glu Lys Leu
            2130                2135                2140

act gct ctc aca aaa aag tat aga att aca gaa aat gat ata caa att      6602
Thr Ala Leu Thr Lys Lys Tyr Arg Ile Thr Glu Asn Asp Ile Gln Ile
        2145                2150                2155

gca tta gat gat gcc aaa atc aac ttt aat gaa aaa cta tct caa ctg      6650
Ala Leu Asp Asp Ala Lys Ile Asn Phe Asn Glu Lys Leu Ser Gln Leu
    2160                2165                2170

cag aca tat atg ata caa ttt gat cag tat att aaa gat agt tat gat      6698
Gln Thr Tyr Met Ile Gln Phe Asp Gln Tyr Ile Lys Asp Ser Tyr Asp
2175                2180                2185                2190

tta cat gat ttg aaa ata gct att gct aat att att gat gaa atc att      6746
Leu His Asp Leu Lys Ile Ala Ile Ala Asn Ile Ile Asp Glu Ile Ile
                2195                2200                2205

gaa aaa tta aaa agt ctt gat gag cac tat cat atc cgt gta aat tta      6794
Glu Lys Leu Lys Ser Leu Asp Glu His Tyr His Ile Arg Val Asn Leu
            2210                2215                2220

gta aaa aca atc cat gat cta cat ttg ttt att gaa aat att gat ttt      6842
Val Lys Thr Ile His Asp Leu His Leu Phe Ile Glu Asn Ile Asp Phe
        2225                2230                2235

aac aaa agt gga agt agt act gca tcc tgg att caa aat gtg gat act      6890
Asn Lys Ser Gly Ser Ser Thr Ala Ser Trp Ile Gln Asn Val Asp Thr
    2240                2245                2250

aag tac caa atc aga atc cag ata caa gaa aaa ctg cag cag ctt aag      6938
Lys Tyr Gln Ile Arg Ile Gln Ile Gln Glu Lys Leu Gln Gln Leu Lys
2255                2260                2265                2270

aga cac ata cag aat ata gac atc cag cac cta gct gga aag tta aaa      6986
Arg His Ile Gln Asn Ile Asp Ile Gln His Leu Ala Gly Lys Leu Lys
                2275                2280                2285
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caa cac att gag gct att gat gtt aga gtg ctt tta gat caa ttg gga    7034
Gln His Ile Glu Ala Ile Asp Val Arg Val Leu Leu Asp Gln Leu Gly
            2290                2295                2300

act aca att tca ttt gaa aga ata aat gat gtt ctt gag cat gtc aaa    7082
Thr Thr Ile Ser Phe Glu Arg Ile Asn Asp Val Leu Glu His Val Lys
        2305                2310                2315

cac ttt gtt ata aat ctt att ggg gat ttt gaa gta gct gag aaa atc    7130
His Phe Val Ile Asn Leu Ile Gly Asp Phe Glu Val Ala Glu Lys Ile
    2320                2325                2330

aat gcc ttc aga gcc aaa gtc cat gag tta atc gag agg tat gaa gta    7178
Asn Ala Phe Arg Ala Lys Val His Glu Leu Ile Glu Arg Tyr Glu Val
2335                2340                2345                2350

gac caa caa atc cag gtt tta atg gat aaa tta gta gag ttg acc cac    7226
Asp Gln Gln Ile Gln Val Leu Met Asp Lys Leu Val Glu Leu Thr His
                2355                2360                2365

caa tac aag ttg aag gag act att cag aag cta agc aat gtc cta caa    7274
Gln Tyr Lys Leu Lys Glu Thr Ile Gln Lys Leu Ser Asn Val Leu Gln
            2370                2375                2380

caa gtt aag ata aaa gat tac ttt gag aaa ttg gtt gga ttt att gat    7322
Gln Val Lys Ile Lys Asp Tyr Phe Glu Lys Leu Val Gly Phe Ile Asp
        2385                2390                2395

gat gct gtg aag aag ctt aat gaa tta tct ttt aaa aca ttc att gaa    7370
Asp Ala Val Lys Lys Leu Asn Glu Leu Ser Phe Lys Thr Phe Ile Glu
    2400                2405                2410

gat gtt aac aaa ttc ctt gac atg ttg ata aag aaa tta aag tca ttt    7418
Asp Val Asn Lys Phe Leu Asp Met Leu Ile Lys Lys Leu Lys Ser Phe
2415                2420                2425                2430

gat tac cac cag ttt gta gat gaa acc aat gac aaa atc cgt gag gtg    7466
Asp Tyr His Gln Phe Val Asp Glu Thr Asn Asp Lys Ile Arg Glu Val
                2435                2440                2445

act cag aga ctc aat ggt gaa att cag gct ctg gaa cta cca caa aaa    7514
Thr Gln Arg Leu Asn Gly Glu Ile Gln Ala Leu Glu Leu Pro Gln Lys
            2450                2455                2460

gct gaa gca tta aaa ctg ttt tta gag gaa acc aag gcc aca gtt gca    7562
Ala Glu Ala Leu Lys Leu Phe Leu Glu Glu Thr Lys Ala Thr Val Ala
        2465                2470                2475

gtg tat ctg gaa agc cta cag gac acc aaa ata acc tta atc atc aat    7610
Val Tyr Leu Glu Ser Leu Gln Asp Thr Lys Ile Thr Leu Ile Ile Asn
    2480                2485                2490

tgg tta cag gag gct tta agt tca gca tct ttg gct cac atg aag gcc    7658
Trp Leu Gln Glu Ala Leu Ser Ser Ala Ser Leu Ala His Met Lys Ala
2495                2500                2505                2510

aaa ttc cga gag act cta gaa gat aca cga gac cga atg tat caa atg    7706
Lys Phe Arg Glu Thr Leu Glu Asp Thr Arg Asp Arg Met Tyr Gln Met
                2515                2520                2525

gac att cag cag gaa ctt caa cga tac ctg tct ctg gta ggc cag gtt    7754
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Asp Ile Gln Gln Glu Leu Gln Arg Tyr Leu Ser Leu Val Gly Gln Val
          2530                2535                2540

tat agc aca ctt gtc acc tac att tct gat tgg tgg act ctt gct gct    7802
Tyr Ser Thr Leu Val Thr Tyr Ile Ser Asp Trp Trp Thr Leu Ala Ala
        2545                2550                2555

aag aac ctt act gac ttt gca gag caa tat tct atc caa gat tgg gct    7850
Lys Asn Leu Thr Asp Phe Ala Glu Gln Tyr Ser Ile Gln Asp Trp Ala
    2560                2565                2570

aaa cgt atg aaa gca ttg gta gag caa ggg ttc act gtt cct gaa atc    7898
Lys Arg Met Lys Ala Leu Val Glu Gln Gly Phe Thr Val Pro Glu Ile
2575                2580                2585                2590

aag acc atc ctt ggg acc atg cct gcc ttt gaa gtc agt ctt cag gct    7946
Lys Thr Ile Leu Gly Thr Met Pro Ala Phe Glu Val Ser Leu Gln Ala
                2595                2600                2605

ctt cag aaa gct acc ttc cag aca cct gat ttt ata gtc ccc cta aca    7994
Leu Gln Lys Ala Thr Phe Gln Thr Pro Asp Phe Ile Val Pro Leu Thr
            2610                2615                2620

gat ttg agg att cca tca gtt cag ata aac ttc aaa gac tta aaa aat    8042
Asp Leu Arg Ile Pro Ser Val Gln Ile Asn Phe Lys Asp Leu Lys Asn
        2625                2630                2635

ata aaa atc cca tcc agg ttt tcc aca cca gaa ttt acc atc ctt aac    8090
Ile Lys Ile Pro Ser Arg Phe Ser Thr Pro Glu Phe Thr Ile Leu Asn
    2640                2645                2650

acc ttc cac att cct tcc ttt aca att gac ttt gtc gaa atg aaa gta    8138
Thr Phe His Ile Pro Ser Phe Thr Ile Asp Phe Val Glu Met Lys Val
2655                2660                2665                2670

aag atc atc aga acc att gac cag atg cag aac agt gag ctg cag tgg    8186
Lys Ile Ile Arg Thr Ile Asp Gln Met Gln Asn Ser Glu Leu Gln Trp
                2675                2680                2685

ccc gtt cca gat ata tat ctc agg gat ctg aag gtg gag gac att cct    8234
Pro Val Pro Asp Ile Tyr Leu Arg Asp Leu Lys Val Glu Asp Ile Pro
            2690                2695                2700

cta gcg aga atc acc ctg cca gac ttc cgt tta cca gaa atc gca att    8282
Leu Ala Arg Ile Thr Leu Pro Asp Phe Arg Leu Pro Glu Ile Ala Ile
        2705                2710                2715

cca gaa ttc ata atc cca act ctc aac ctt aat gat ttt caa gtt cct    8330
Pro Glu Phe Ile Ile Pro Thr Leu Asn Leu Asn Asp Phe Gln Val Pro
    2720                2725                2730

gac ctt cac ata cca gaa ttc cag ctt ccc cac atc tca cac aca att    8378
Asp Leu His Ile Pro Glu Phe Gln Leu Pro His Ile Ser His Thr Ile
2735                2740                2745                2750

gaa gta cct act ttt ggc aag cta tac agt att ctg aaa atc caa tct    8426
Glu Val Pro Thr Phe Gly Lys Leu Tyr Ser Ile Leu Lys Ile Gln Ser
                2755                2760                2765

cct ctt ttc aca tta gat gca aat gct gac ata ggg aat gga acc acc    8474
Pro Leu Phe Thr Leu Asp Ala Asn Ala Asp Ile Gly Asn Gly Thr Thr
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2770 2775 2780

tca gca aac gaa gca ggt atc gca gct tcc atc act gcc aaa gga gag 8522
Ser Ala Asn Glu Ala Gly Ile Ala Ala Ser Ile Thr Ala Lys Gly Glu
2785 2790 2795

tcc aaa tta gaa gtt ctc aat ttt gat ttt caa gca aat gca caa ctc 8570
Ser Lys Leu Glu Val Leu Asn Phe Asp Phe Gln Ala Asn Ala Gln Leu
2800 2805 2810

tca aac cct aag att aat ccg ctg gct ctg aag gag tca gtg aag ttc 8618
Ser Asn Pro Lys Ile Asn Pro Leu Ala Leu Lys Glu Ser Val Lys Phe
2815 2820 2825 2830

tcc agc aag tac ctg aga acg gag cat ggg agt gaa atg ctg ttt ttt 8666
Ser Ser Lys Tyr Leu Arg Thr Glu His Gly Ser Glu Met Leu Phe Phe
2835 2840 2845

gga aat gct att gag gga aaa tca aac aca gtg gca agt tta cac aca 8714
Gly Asn Ala Ile Glu Gly Lys Ser Asn Thr Val Ala Ser Leu His Thr
2850 2855 2860

gaa aaa aat aca ctg gag ctt agt aat gga gtg att gtc aag ata aac 8762
Glu Lys Asn Thr Leu Glu Leu Ser Asn Gly Val Ile Val Lys Ile Asn
2865 2870 2875

aat cag ctt acc ctg gat agc aac act aaa tac ttc cac aaa ttg aac 8810
Asn Gln Leu Thr Leu Asp Ser Asn Thr Lys Tyr Phe His Lys Leu Asn
2880 2885 2890

atc ccc aaa ctg gac ttc tct agt cag gct gac ctg cgc aac gag atc 8858
Ile Pro Lys Leu Asp Phe Ser Ser Gln Ala Asp Leu Arg Asn Glu Ile
2895 2900 2905 2910

aag aca ctg ttg aaa gct ggc cac ata gca tgg act tct tct gga aaa 8906
Lys Thr Leu Leu Lys Ala Gly His Ile Ala Trp Thr Ser Ser Gly Lys
2915 2920 2925

ggg tca tgg aaa tgg gcc tgc ccc aga ttc tca gat gag gga aca cat 8954
Gly Ser Trp Lys Trp Ala Cys Pro Arg Phe Ser Asp Glu Gly Thr His
2930 2935 2940

gaa tca caa att agt ttc acc ata gaa gga ccc ctc act tcc ttt gga 9002
Glu Ser Gln Ile Ser Phe Thr Ile Glu Gly Pro Leu Thr Ser Phe Gly
2945 2950 2955

ctg tcc aat aag atc aat agc aaa cac cta aga gta aac caa aac ttg 9050
Leu Ser Asn Lys Ile Asn Ser Lys His Leu Arg Val Asn Gln Asn Leu
2960 2965 2970

gtt tat gaa tct ggc tcc ctc aac ttt tct aaa ctt gaa att caa tca 9098
Val Tyr Glu Ser Gly Ser Leu Asn Phe Ser Lys Leu Glu Ile Gln Ser
2975 2980 2985 2990

caa gtc gat tcc cag cat gtg ggc cac agt gtt cta act gct aaa ggc 9146
Gln Val Asp Ser Gln His Val Gly His Ser Val Leu Thr Ala Lys Gly
2995 3000 3005

atg gca ctg ttt gga gaa ggg aag gca gag ttt act ggg agg cat gat 9194
Met Ala Leu Phe Gly Glu Gly Lys Ala Glu Phe Thr Gly Arg His Asp
3010 3015 3020

gct cat tta aat gga aag gtt att gga act ttg aaa aat tct ctt ttc 9242

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Ala His Leu Asn Gly Lys Val Ile Gly Thr Leu Lys Asn Ser Leu Phe
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ttt tca gcc cag cca ttt gag atc acg gca tcc aca aac aat gaa ggg      9290
Phe Ser Ala Gln Pro Phe Glu Ile Thr Ala Ser Thr Asn Asn Glu Gly
    3040                3045                3050

aat ttg aaa gtt cgt ttt cca tta agg tta aca ggg aag ata gac ttc      9338
Asn Leu Lys Val Arg Phe Pro Leu Arg Leu Thr Gly Lys Ile Asp Phe
3055                3060                3065                3070

ctg aat aac tat gca ctg ttt ctg agt ccc agt gcc cag caa gca agt      9386
Leu Asn Asn Tyr Ala Leu Phe Leu Ser Pro Ser Ala Gln Gln Ala Ser
                3075                3080                3085

tgg caa gta agt gct agg ttc aat cag tat aag tac aac caa aat ttc      9434
Trp Gln Val Ser Ala Arg Phe Asn Gln Tyr Lys Tyr Asn Gln Asn Phe
            3090                3095                3100

tct gct gga aac aac gag aac att atg gag gcc cat gta gga ata aat      9482
Ser Ala Gly Asn Asn Glu Asn Ile Met Glu Ala His Val Gly Ile Asn
        3105                3110                3115

gga gaa gca aat ctg gat ttc tta aac att cct tta aca att cct gaa      9530
Gly Glu Ala Asn Leu Asp Phe Leu Asn Ile Pro Leu Thr Ile Pro Glu
    3120                3125                3130

atg cgt cta cct tac aca ata atc aca act cct cca ctg aaa gat ttc      9578
Met Arg Leu Pro Tyr Thr Ile Ile Thr Thr Pro Pro Leu Lys Asp Phe
3135                3140                3145                3150

tct cta tgg gaa aaa aca ggc ttg aag gaa ttc ttg aaa acg aca aag      9626
Ser Leu Trp Glu Lys Thr Gly Leu Lys Glu Phe Leu Lys Thr Thr Lys
                3155                3160                3165

caa tca ttt gat tta agt gta aaa gct cag tat aag aaa aac aaa cac      9674
Gln Ser Phe Asp Leu Ser Val Lys Ala Gln Tyr Lys Lys Asn Lys His
            3170                3175                3180

agg cat tcc atc aca aat cct ttg gct gtg ctt tgt gag ttt atc agt      9722
Arg His Ser Ile Thr Asn Pro Leu Ala Val Leu Cys Glu Phe Ile Ser
        3185                3190                3195

cag agc atc aaa tcc ttt gac agg cat ttt gaa aaa aac aga aac aat      9770
Gln Ser Ile Lys Ser Phe Asp Arg His Phe Glu Lys Asn Arg Asn Asn
    3200                3205                3210

gca tta gat ttt gtc acc aaa tcc tat aat gaa aca aaa att aag ttt      9818
Ala Leu Asp Phe Val Thr Lys Ser Tyr Asn Glu Thr Lys Ile Lys Phe
3215                3220                3225                3230

gat aag tac aaa gct gaa aaa tct cac gac gag ctc ccc agg acc ttt      9866
Asp Lys Tyr Lys Ala Glu Lys Ser His Asp Glu Leu Pro Arg Thr Phe
                3235                3240                3245

caa att cct gga tac act gtt cca gtt gtc aat gtt gaa gtg tct cca      9914
Gln Ile Pro Gly Tyr Thr Val Pro Val Val Asn Val Glu Val Ser Pro
            3250                3255                3260

ttc acc ata gag atg tcg gca ttc ggc tat gtg ttc cca aaa gca gtc      9962
Phe Thr Ile Glu Met Ser Ala Phe Gly Tyr Val Phe Pro Lys Ala Val
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        3265                3270                3275

agc atg cct agt ttc tcc atc cta ggt tct gac gtc cgt gtg cct tca    10010
Ser Met Pro Ser Phe Ser Ile Leu Gly Ser Asp Val Arg Val Pro Ser
    3280                3285                3290

tac aca tta atc ctg cca tca tta gag ctg cca gtc ctt cat gtc cct    10058
Tyr Thr Leu Ile Leu Pro Ser Leu Glu Leu Pro Val Leu His Val Pro
3295                3300                3305                3310

aga aat ctc aag ctt tct ctt cca cat ttc aag gaa ttg tgt acc ata    10106
Arg Asn Leu Lys Leu Ser Leu Pro His Phe Lys Glu Leu Cys Thr Ile
                3315                3320                3325

agc cat att ttt att cct gcc atg ggc aat att acc tat gat ttc tcc    10154
Ser His Ile Phe Ile Pro Ala Met Gly Asn Ile Thr Tyr Asp Phe Ser
            3330                3335                3340

ttt aaa tca agt gtc atc aca ctg aat acc aat gct gaa ctt ttt aac    10202
Phe Lys Ser Ser Val Ile Thr Leu Asn Thr Asn Ala Glu Leu Phe Asn
        3345                3350                3355

cag tca gat att gtt gct cat ctc ctt tct tca tct tca tct gtc att    10250
Gln Ser Asp Ile Val Ala His Leu Leu Ser Ser Ser Ser Ser Val Ile
    3360                3365                3370

gat gca ctg cag tac aaa tta gag ggc acc aca aga ttg aca aga aaa    10298
Asp Ala Leu Gln Tyr Lys Leu Glu Gly Thr Thr Arg Leu Thr Arg Lys
3375                3380                3385                3390

agg gga ttg aag tta gcc aca gct ctg tct ctg agc aac aaa ttt gtg    10346
Arg Gly Leu Lys Leu Ala Thr Ala Leu Ser Leu Ser Asn Lys Phe Val
                3395                3400                3405

gag ggt agt cat aac agt act gtg agc tta acc acg aaa aat atg gaa    10394
Glu Gly Ser His Asn Ser Thr Val Ser Leu Thr Thr Lys Asn Met Glu
            3410                3415                3420

gtg tca gtg gca aaa acc aca aaa gcc gaa att cca att ttg aga atg    10442
Val Ser Val Ala Lys Thr Thr Lys Ala Glu Ile Pro Ile Leu Arg Met
        3425                3430                3435

aat ttc aag caa gaa ctt aat gga aat acc aag tca aaa cct act gtc    10490
Asn Phe Lys Gln Glu Leu Asn Gly Asn Thr Lys Ser Lys Pro Thr Val
    3440                3445                3450

tct tcc tcc atg gaa ttt aag tat gat ttc aat tct tca atg ctg tac    10538
Ser Ser Ser Met Glu Phe Lys Tyr Asp Phe Asn Ser Ser Met Leu Tyr
3455                3460                3465                3470

tct acc gct aaa gga gca gtt gac cac aag ctt agc ttg gaa agc ctc    10586
Ser Thr Ala Lys Gly Ala Val Asp His Lys Leu Ser Leu Glu Ser Leu
                3475                3480                3485

acc tct tac ttt tcc att gag tca tct acc aaa gga gat gtc aag ggt    10634
Thr Ser Tyr Phe Ser Ile Glu Ser Ser Thr Lys Gly Asp Val Lys Gly
            3490                3495                3500

tcg gtt ctt tct cgg gaa tat tca gga act att gct agt gag gcc aac    10682
Ser Val Leu Ser Arg Glu Tyr Ser Gly Thr Ile Ala Ser Glu Ala Asn
        3505                3510                3515

act tac ttg aat tcc aag agc aca cgg tct tca gtg aag ctg cag ggc    10730
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Thr Tyr Leu Asn Ser Lys Ser Thr Arg Ser Ser Val Lys Leu Gln Gly
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act tcc aaa att gat gat atc tgg aac ctt gaa gta aaa gaa aat ttt   10778
Thr Ser Lys Ile Asp Asp Ile Trp Asn Leu Glu Val Lys Glu Asn Phe
3535                3540                3545                3550

gct gga gaa gcc aca ctc caa cgc ata tat tcc ctc tgg gag cac agt   10826
Ala Gly Glu Ala Thr Leu Gln Arg Ile Tyr Ser Leu Trp Glu His Ser
                3555                3560                3565

acg aaa aac cac tta cag cta gag ggc ctc ttt ttc acc aac gga gaa   10874
Thr Lys Asn His Leu Gln Leu Glu Gly Leu Phe Phe Thr Asn Gly Glu
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cat aca agc aaa gcc acc ctg gaa ctc tct cca tgg caa atg tca gct   10922
His Thr Ser Lys Ala Thr Leu Glu Leu Ser Pro Trp Gln Met Ser Ala
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ctt gtt cag gtc cat gca agt cag ccc agt tcc ttc cat gat ttc cct   10970
Leu Val Gln Val His Ala Ser Gln Pro Ser Ser Phe His Asp Phe Pro
    3600                3605                3610

gac ctt ggc cag gaa gtg gcc ctg aat gct aac act aag aac cag aag   11018
Asp Leu Gly Gln Glu Val Ala Leu Asn Ala Asn Thr Lys Asn Gln Lys
3615                3620                3625                3630

atc aga tgg aaa aat gaa gtc cgg att cat tct ggg tct ttc cag agc   11066
Ile Arg Trp Lys Asn Glu Val Arg Ile His Ser Gly Ser Phe Gln Ser
                3635                3640                3645

cag gtc gag ctt tcc aat gac caa gaa aag gca cac ctt gac att gca   11114
Gln Val Glu Leu Ser Asn Asp Gln Glu Lys Ala His Leu Asp Ile Ala
            3650                3655                3660

gga tcc tta gaa gga cac cta agg ttc ctc aaa aat atc atc cta cca   11162
Gly Ser Leu Glu Gly His Leu Arg Phe Leu Lys Asn Ile Ile Leu Pro
        3665                3670                3675

gtc tat gac aag agc tta tgg gat ttc cta aag ctg gat gta acc acc   11210
Val Tyr Asp Lys Ser Leu Trp Asp Phe Leu Lys Leu Asp Val Thr Thr
    3680                3685                3690

agc att ggt agg aga cag cat ctt cgt gtt tca act gcc ttt gtg tac   11258
Ser Ile Gly Arg Arg Gln His Leu Arg Val Ser Thr Ala Phe Val Tyr
3695                3700                3705                3710

acc aaa aac ccc aat ggc tat tca ttc tcc atc cct gta aaa gtt ttg   11306
Thr Lys Asn Pro Asn Gly Tyr Ser Phe Ser Ile Pro Val Lys Val Leu
                3715                3720                3725

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Ala Asp Lys Phe Ile Thr Pro Gly Leu Lys Leu Asn Asp Leu Asn Ser
            3730                3735                3740

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Val Leu Val Met Pro Thr Phe His Val Pro Phe Thr Asp Leu Gln Val
        3745                3750                3755
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cca tcg tgc aaa ctt gac ttc aga gaa ata caa atc tat aag aag ctg 11450
Pro Ser Cys Lys Leu Asp Phe Arg Glu Ile Gln Ile Tyr Lys Lys Leu
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3775 3780 3785 3790

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Phe Pro Glu Val Asp Val Leu Thr Lys Tyr Ser Gln Pro Glu Asp Ser
3795 3800 3805

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Ser Gln Phe Thr Leu Pro Lys Ser Val Ser Asp Gly Ile Ala Ala Leu
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3840 3845 3850

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Ile Ile Val Pro Glu Gln Thr Ile Glu Ile Pro Ser Ile Lys Phe Ser
3855 3860 3865 3870

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Val Pro Ala Gly Ile Val Ile Pro Ser Phe Gln Ala Leu Thr Ala Arg
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Phe Glu Val Asp Ser Pro Val Tyr Asn Ala Thr Trp Ser Ala Ser Leu
3890 3895 3900

aaa aac aaa gca gat tat gtt gaa aca gtc ctg gat tcc aca tgc agc 11882
Lys Asn Lys Ala Asp Tyr Val Glu Thr Val Leu Asp Ser Thr Cys Ser
3905 3910 3915

tca acc gta cag ttc cta gaa tat gaa cta aat gtt ttg gga aca cac 11930
Ser Thr Val Gln Phe Leu Glu Tyr Glu Leu Asn Val Leu Gly Thr His
3920 3925 3930

aaa atc gaa gat ggt acg tta gcc tct aag act aaa gga aca ctt gca 11978
Lys Ile Glu Asp Gly Thr Leu Ala Ser Lys Thr Lys Gly Thr Leu Ala
3935 3940 3945 3950

cac cgt gac ttc agt gca gaa tat gaa gaa gat ggc aaa ttt gaa gga 12026
His Arg Asp Phe Ser Ala Glu Tyr Glu Glu Asp Gly Lys Phe Glu Gly
3955 3960 3965

ctt cag gaa tgg gaa gga aaa gcg cac ctc aat atc aaa agc cca gcg 12074
Leu Gln Glu Trp Glu Gly Lys Ala His Leu Asn Ile Lys Ser Pro Ala
3970 3975 3980

ttc acc gat ctc cat ctg cgc tac cag aaa gac aag aaa ggc atc tcc 12122
Phe Thr Asp Leu His Leu Arg Tyr Gln Lys Asp Lys Lys Gly Ile Ser
3985 3990 3995

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Thr Ser Ala Ala Ser Pro Ala Val Gly Thr Val Gly Met Asp Met Asp

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gaa gat gac gac ttt tct aaa tgg aac ttc tac tac agc cct cag tcc    12218
Glu Asp Asp Asp Phe Ser Lys Trp Asn Phe Tyr Tyr Ser Pro Gln Ser
4015                4020                4025                4030

tct cca gat aaa aaa ctc acc ata ttc aaa act gag ttg agg gtc cgg    12266
Ser Pro Asp Lys Lys Leu Thr Ile Phe Lys Thr Glu Leu Arg Val Arg
                4035                4040                4045

gaa tct gat gag gaa act cag atc aaa gtt aat tgg gaa gaa gag gca    12314
Glu Ser Asp Glu Glu Thr Gln Ile Lys Val Asn Trp Glu Glu Glu Ala
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gct tct ggc ttg cta acc tct ctg aaa gac aac gtg ccc aag gcc aca    12362
Ala Ser Gly Leu Leu Thr Ser Leu Lys Asp Asn Val Pro Lys Ala Thr
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ggg gtc ctt tat gat tat gtc aac aag tac cac tgg gaa cac aca ggg    12410
Gly Val Leu Tyr Asp Tyr Val Asn Lys Tyr His Trp Glu His Thr Gly
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ctc acc ctg aga gaa gtg tct tca aag ctg aga aga aat ctg cag aac    12458
Leu Thr Leu Arg Glu Val Ser Ser Lys Leu Arg Arg Asn Leu Gln Asn
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Asn Ala Glu Trp Val Tyr Gln Gly Ala Ile Arg Gln Ile Asp Asp Ile
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Asp Val Arg Phe Gln Lys Ala Ala Ser Gly Thr Thr Gly Thr Tyr Gln
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Glu Trp Lys Asp Lys Ala Gln Asn Leu Tyr Gln Glu Leu Leu Thr Gln
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Glu Gly Gln Ala Ser Phe Gln Gly Leu Lys Asp Asn Val Phe Asp Gly
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Leu Val Arg Val Thr Gln Lys Phe His Met Lys Val Lys His Leu Ile
4175                4180                4185                4190

gac tca ctc att gat ttt ctg aac ttc ccc aga ttc cag ttt ccg ggg    12746
Asp Ser Leu Ile Asp Phe Leu Asn Phe Pro Arg Phe Gln Phe Pro Gly
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Lys Pro Gly Ile Tyr Thr Arg Glu Glu Leu Cys Thr Met Phe Ile Arg
            4210                4215                4220

gag gta ggg acg gta ctg tcc cag gta tat tcg aaa gtc cat aat ggt    12842
Glu Val Gly Thr Val Leu Ser Gln Val Tyr Ser Lys Val His Asn Gly
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tca gaa ata ctg ttt tcc tat ttc caa gac cta gtg att aca ctt cct    12890
Ser Glu Ile Leu Phe Ser Tyr Phe Gln Asp Leu Val Ile Thr Leu Pro
    4240                4245                4250

ttc gag tta agg aaa cat aaa cta ata gat gta atc tcg atg tat agg    12938
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Phe Glu Leu Arg Lys His Lys Leu Ile Asp Val Ile Ser Met Tyr Arg
4255 4260 4265 4270

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Glu Leu Leu Lys Asp Leu Ser Lys Glu Ala Gln Glu Val Phe Lys Ala
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Ile Gln Ser Leu Lys Thr Thr Glu Val Leu Arg Asn Leu Gln Asp Leu
4290 4295 4300

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Glu Met Lys Phe Thr Tyr Leu Ile Asn Tyr Ile Gln Asp Glu Ile Asn
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Thr Ile Phe Asn Asp Tyr Ile Pro Tyr Val Phe Lys Leu Leu Lys Glu
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Lys Tyr Tyr Glu Leu Glu Glu Lys Ile Val Ser Leu Ile Lys Asn Leu
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Lys Glu Lys Ile Ala Glu Leu Ser Ala Thr Ala Gln Glu Ile Ile Lys
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Ser Gln Ala Ile Ala Thr Lys Lys Ile Ile Ser Asp Tyr His Gln Gln
4480 4485 4490

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Phe Arg Tyr Lys Leu Gln Asp Phe Ser Asp Gln Leu Ser Asp Tyr Tyr

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Glu Lys Phe Ile Ala Glu Ser Lys Arg Leu Ile Asp Leu Ser Ile Gln
                4515                4520                4525

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Asn Tyr His Thr Phe Leu Ile Tyr Ile Thr Glu Leu Leu Lys Lys Leu
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Gln Ser Thr Thr Val Met Asn Pro Tyr Met Lys Leu Ala Pro Gly Glu
        4545                4550                4555

ctt act atc atc ctc taa tttttaaaa gaaatcttca tttattcttc            13850
Leu Thr Ile Ile Leu  *
    4560

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gtaacaactt cagggaacaa agcagagatt gtggcttctg tcactgctaa aggagagtcc 180

caatttgaag ctctcaattt tgattttcaa gcacaagctc aattcctgga gttaaatcct 240

catcctccag tcctgaagga atccatgaac ttctccagta agcatgtgag aatggagcat 300

gagggtgaga tagtatttga tggaaaggcc attgagggga aatcagacac agtcgcaagt 360

ttacacacag agaaaaatga agtagagttt aataatggta tgactgtcaa agtaaacaat 420

cagctcaccc ttgacagtca cacaaagtac ttccacaagt tgagtgttcc taggctggac 480

ttctccagta aggcttctct taataatgaa atcaagacac tattagaagc tggacatgtg 540

gcattgacat cttcagggac agggtcatgg aactgggcct gtcccaactt ctcggatgaa 600

ggcatacatt cgtcccaaat tagctttact gtggatggtc ccattgcttt tgttggacta 660

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Leu Leu Leu Leu Leu Leu Ala Gly Ala Arg Ala Glu Glu Glu Met Leu
20 25 30

gaa aat gtc agc ctg gtc tgt cca aaa gat gcg acc cga ttc aag cac 144
Glu Asn Val Ser Leu Val Cys Pro Lys Asp Ala Thr Arg Phe Lys His
35 40 45

ctc cgg aag tac aca tac aac tat gag gct gag agt tcc agt gga gtc 192
Leu Arg Lys Tyr Thr Tyr Asn Tyr Glu Ala Glu Ser Ser Ser Gly Val
50 55 60

cct ggg act gct gat tca aga agt gcc acc agg atc aac tgc aag gtt 240
Pro Gly Thr Ala Asp Ser Arg Ser Ala Thr Arg Ile Asn Cys Lys Val
65 70 75 80

gag ctg gag gtt ccc cag ctc tgc agc ttc atc ctg aag acc agc cag 288
Glu Leu Glu Val Pro Gln Leu Cys Ser Phe Ile Leu Lys Thr Ser Gln
85 90 95

tgc acc ctg aaa gag gtg tat ggc ttc aac cct gag ggc aaa gcc ttg 336
Cys Thr Leu Lys Glu Val Tyr Gly Phe Asn Pro Glu Gly Lys Ala Leu
100 105 110

ctg aag aaa acc aag aac tct gag gag ttt gct gca gcc atg tcc agg 384
Leu Lys Lys Thr Lys Asn Ser Glu Glu Phe Ala Ala Ala Met Ser Arg
115 120 125

tat gag ctc aag ctg gcc att cca gaa ggg aag cag gtt ttc ctt tac 432
Tyr Glu Leu Lys Leu Ala Ile Pro Glu Gly Lys Gln Val Phe Leu Tyr
130 135 140

ccg gag aaa gat gaa cct act tac atc ctg aac atc aag agg ggc atc 480
Pro Glu Lys Asp Glu Pro Thr Tyr Ile Leu Asn Ile Lys Arg Gly Ile
145 150 155 160

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att tct gcc ctc ctg gtt ccc cca gag aca gaa gaa gcc aag caa gtg    528
Ile Ser Ala Leu Leu Val Pro Pro Glu Thr Glu Glu Ala Lys Gln Val
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ttg ttt ctg gat acc gtg tat gga aac tgc tcc act cac ttt acc gtc    576
Leu Phe Leu Asp Thr Val Tyr Gly Asn Cys Ser Thr His Phe Thr Val
            180                 185                 190

aag acg agg aag ggc aat gtg gca aca gaa ata tcc act gaa aga gac    624
Lys Thr Arg Lys Gly Asn Val Ala Thr Glu Ile Ser Thr Glu Arg Asp
        195                 200                 205

ctg ggg cag tgt gat cgc ttc aag ccc atc cgc aca ggc atc agc cca    672
Leu Gly Gln Cys Asp Arg Phe Lys Pro Ile Arg Thr Gly Ile Ser Pro
    210                 215                 220

ctt gct ctc atc aaa ggc atg acc cgc ccc ttg tca act ctg atc agc    720
Leu Ala Leu Ile Lys Gly Met Thr Arg Pro Leu Ser Thr Leu Ile Ser
225                 230                 235                 240

agc agc cag tcc tgt cag tac aca ctg gac gct aag agg aag cat gtg    768
Ser Ser Gln Ser Cys Gln Tyr Thr Leu Asp Ala Lys Arg Lys His Val
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gca gaa gcc atc tgc aag gag caa cac ctc ttc ctg cct ttc tcc tac    816
Ala Glu Ala Ile Cys Lys Glu Gln His Leu Phe Leu Pro Phe Ser Tyr
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aag aat aag tat ggg atg gta gca caa gtg aca cag act ttg aaa ctt    864
Lys Asn Lys Tyr Gly Met Val Ala Gln Val Thr Gln Thr Leu Lys Leu
        275                 280                 285

gaa gac aca cca aag atc aac agc cgc ttc ttt ggt gaa ggt act aag    912
Glu Asp Thr Pro Lys Ile Asn Ser Arg Phe Phe Gly Glu Gly Thr Lys
    290                 295                 300

aag atg ggc ctc gca ttt gag agc acc aaa tcc aca tca cct cca aag    960
Lys Met Gly Leu Ala Phe Glu Ser Thr Lys Ser Thr Ser Pro Pro Lys
305                 310                 315                 320

cag gcc gaa gct gtt ttg aag act ctc cag gaa ctg aaa aaa cta acc    1008
Gln Ala Glu Ala Val Leu Lys Thr Leu Gln Glu Leu Lys Lys Leu Thr
                325                 330                 335

atc tct gag caa aat atc cag aga gct aat ctc ttc aat aag ctg gtt    1056
Ile Ser Glu Gln Asn Ile Gln Arg Ala Asn Leu Phe Asn Lys Leu Val
            340                 345                 350

act gag ctg aga ggc ctc agt gat gaa gca gtc aca tct ctc ttg cca    1104
Thr Glu Leu Arg Gly Leu Ser Asp Glu Ala Val Thr Ser Leu Leu Pro
        355                 360                 365

cag ctg att gag gtg tcc agc ccc atc act tta caa gcc ttg gtt cag    1152
Gln Leu Ile Glu Val Ser Ser Pro Ile Thr Leu Gln Ala Leu Val Gln
    370                 375                 380

tgt gga cag cct cag tgc tcc act cac atc ctc cag tgg ctg aaa cgt    1200
Cys Gly Gln Pro Gln Cys Ser Thr His Ile Leu Gln Trp Leu Lys Arg
385                 390                 395                 400
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405 410 415

ctg atc ccc gag ccc tca gca cag cag ctg cga gag atc ttc aac atg 1296
Leu Ile Pro Glu Pro Ser Ala Gln Gln Leu Arg Glu Ile Phe Asn Met
420 425 430

gcg agg gat cag cgc agc cga gcc acc ttg tat gcg ctg agc cac gcg 1344
Ala Arg Asp Gln Arg Ser Arg Ala Thr Leu Tyr Ala Leu Ser His Ala
435 440 445

gtc aac aac tat cat aag aca aac cct aca ggg acc cag gag ctg ctg 1392
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450 455 460

gac att gct aat tac ctg atg gaa cag att caa gat gac tgc act ggg 1440
Asp Ile Ala Asn Tyr Leu Met Glu Gln Ile Gln Asp Asp Cys Thr Gly
465 470 475 480

gat gaa gat tac acc tat ttg att ctg cgg gtc att gga aat atg ggc 1488
Asp Glu Asp Tyr Thr Tyr Leu Ile Leu Arg Val Ile Gly Asn Met Gly
485 490 495

caa acc atg gag cag tta act cca gaa ctc aag tct tca atc ctg aaa 1536
Gln Thr Met Glu Gln Leu Thr Pro Glu Leu Lys Ser Ser Ile Leu Lys
500 505 510

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Cys Val Gln Ser Thr Lys Pro Ser Leu Met Ile Gln Lys Ala Ala Ile
515 520 525

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Gln Ala Leu Arg Lys Met Glu Pro Lys Asp Lys Asp Gln Glu Val Leu
530 535 540

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Leu Gln Thr Phe Leu Asp Asp Ala Ser Pro Gly Asp Lys Arg Leu Ala
545 550 555 560

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Ala Tyr Leu Met Leu Met Arg Ser Pro Ser Gln Ala Asp Ile Asn Lys
565 570 575

att gtc caa att cta cca tgg gaa cag aat gag caa gtg aag aac ttt 1776
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Val Ala Ser His Ile Ala Asn Ile Leu Asn Ser Glu Glu Leu Asp Ile
595 600 605

caa gat ctg aaa aag tta gtg aaa gaa gct ctg aaa gaa tct caa ctt 1872
Gln Asp Leu Lys Lys Leu Val Lys Glu Ala Leu Lys Glu Ser Gln Leu
610 615 620

cca act gtc atg gac ttc aga aaa ttc tct cgg aac tat caa ctc tac 1920
Pro Thr Val Met Asp Phe Arg Lys Phe Ser Arg Asn Tyr Gln Leu Tyr
625 630 635 640

aaa tct gtt tct ctt cca tca ctt gac cca gcc tca gcc aaa ata gaa 1968
Lys Ser Val Ser Leu Pro Ser Leu Asp Pro Ala Ser Ala Lys Ile Glu
645 650 655

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ctg aaa act acc ctc act gcc ttt gga ttt gct tca gct gac ctc atc    2064
Leu Lys Thr Thr Leu Thr Ala Phe Gly Phe Ala Ser Ala Asp Leu Ile
        675                 680                 685

gag att ggc ttg gaa gga aaa ggc ttt gag cca aca ttg gag gct cct    2112
Glu Ile Gly Leu Glu Gly Lys Gly Phe Glu Pro Thr Leu Glu Ala Pro
    690                 695                 700

ttt ggg aag caa gga ttt ttc cca gac agt gtc aac aaa gct ttg tac    2160
Phe Gly Lys Gln Gly Phe Phe Pro Asp Ser Val Asn Lys Ala Leu Tyr
705                 710                 715                 720

tgg gtt aat ggt caa gtt cct gat ggt gtc tct aag gtc tta gtg gac    2208
Trp Val Asn Gly Gln Val Pro Asp Gly Val Ser Lys Val Leu Val Asp
                725                 730                 735

cac ttt ggc tat acc aaa gat gat aaa cat gag cag gat atg gta aat    2256
His Phe Gly Tyr Thr Lys Asp Asp Lys His Glu Gln Asp Met Val Asn
            740                 745                 750

gga ata atg ctc agt gtt gag aag ctg att aaa gat ttg aaa tcc aaa    2304
Gly Ile Met Leu Ser Val Glu Lys Leu Ile Lys Asp Leu Lys Ser Lys
        755                 760                 765

gaa gtc ccg gaa gcc aga gcc tac ctc cgc atc ttg gga gag gag ctt    2352
Glu Val Pro Glu Ala Arg Ala Tyr Leu Arg Ile Leu Gly Glu Glu Leu
    770                 775                 780

ggt ttt gcc agt ctc cat gac ctc cga ctc ctg gga aag ctg ctt ctg    2400
Gly Phe Ala Ser Leu His Asp Leu Arg Leu Leu Gly Lys Leu Leu Leu
785                 790                 795                 800

atg ggt gcc cgc act ctg cag ggg atc ccc cag atg att gga gag gtc    2448
Met Gly Ala Arg Thr Leu Gln Gly Ile Pro Gln Met Ile Gly Glu Val
                805                 810                 815

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Ile Arg Lys Gly Ser Lys Asn Asp Phe Phe Leu His Tyr Ile Phe Met
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gag aat gcc ttt gaa ctc ccc act gga gct gga tta cag ttg caa ata    2544
Glu Asn Ala Phe Glu Leu Pro Thr Gly Ala Gly Leu Gln Leu Gln Ile
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tct tca tct gga gtc att gct ccc gga gcc aag gct gga gta aaa ctg    2592
Ser Ser Ser Gly Val Ile Ala Pro Gly Ala Lys Ala Gly Val Lys Leu
    850                 855                 860

gaa gta gcc aac atg cag gct gaa ctg gtg gca aaa ccc tcc gtg tct    2640
Glu Val Ala Asn Met Gln Ala Glu Leu Val Ala Lys Pro Ser Val Ser
865                 870                 875                 880

gtg gag ttt gtg aca aat atg ggc atc atc att ccg gac ttc gct agg    2688
Val Glu Phe Val Thr Asn Met Gly Ile Ile Ile Pro Asp Phe Ala Arg
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gct cat gtt gcc cta aaa gct ggg aag ctg aag ttt atc att cct tcc     2784
Ala His Val Ala Leu Lys Ala Gly Lys Leu Lys Phe Ile Ile Pro Ser
        915                 920                 925

cca aag aga cca gtc aag ctg ctc agt gga ggc aac aca tta cat ttg     2832
Pro Lys Arg Pro Val Lys Leu Leu Ser Gly Gly Asn Thr Leu His Leu
    930                 935                 940

gtc tct acc acc aaa acg gag gtc atc cca cct ctc att gag aac agg     2880
Val Ser Thr Thr Lys Thr Glu Val Ile Pro Pro Leu Ile Glu Asn Arg
945                 950                 955                 960

cag tcc tgg tca gtt tgc aag caa gtc ttt cct ggc ctg aat tac tgc     2928
Gln Ser Trp Ser Val Cys Lys Gln Val Phe Pro Gly Leu Asn Tyr Cys
                965                 970                 975

acc tca ggc gct tac tcc aac gcc agc tcc aca gac tcc gcc tcc tac     2976
Thr Ser Gly Ala Tyr Ser Asn Ala Ser Ser Thr Asp Ser Ala Ser Tyr
            980                 985                 990

tat ccg ctg acc ggg gac acc aga tta gag ctg gaa ctg agg cct aca     3024
Tyr Pro Leu Thr Gly Asp Thr Arg Leu Glu Leu Glu Leu Arg Pro Thr
        995                 1000                1005

gga gag att gag cag tat tct gtc agc gca acc tat gag ctc cag aga     3072
Gly Glu Ile Glu Gln Tyr Ser Val Ser Ala Thr Tyr Glu Leu Gln Arg
    1010                1015                1020

gag gac aga gcc ttg gtg gat acc ctg aag ttt gta act caa gca gaa     3120
Glu Asp Arg Ala Leu Val Asp Thr Leu Lys Phe Val Thr Gln Ala Glu
1025                1030                1035                1040

ggc gcg aag cag act gag gct acc atg aca ttc aaa tat aat cgg cag     3168
Gly Ala Lys Gln Thr Glu Ala Thr Met Thr Phe Lys Tyr Asn Arg Gln
                1045                1050                1055

agt atg acc ttg tcc agt gaa gtc caa att ccg gat ttt gat gtt gac     3216
Ser Met Thr Leu Ser Ser Glu Val Gln Ile Pro Asp Phe Asp Val Asp
            1060                1065                1070

ctc gga aca atc ctc aga gtt aat gat gaa tct act gag ggc aaa acg     3264
Leu Gly Thr Ile Leu Arg Val Asn Asp Glu Ser Thr Glu Gly Lys Thr
        1075                1080                1085

tct tac aga ctc acc ctg gac att cag aac aag aaa att act gag gtc     3312
Ser Tyr Arg Leu Thr Leu Asp Ile Gln Asn Lys Lys Ile Thr Glu Val
    1090                1095                1100

gcc ctc atg ggc cac cta agt tgt gac aca aag gaa gaa aga aaa atc     3360
Ala Leu Met Gly His Leu Ser Cys Asp Thr Lys Glu Glu Arg Lys Ile
1105                1110                1115                1120

aag ggt gtt att tcc ata ccc cgt ttg caa gca gaa gcc aga agt gag     3408
Lys Gly Val Ile Ser Ile Pro Arg Leu Gln Ala Glu Ala Arg Ser Glu
                1125                1130                1135

atc ctc gcc cac tgg tcg cct gcc aaa ctg ctt ctc caa atg gac tca     3456
Ile Leu Ala His Trp Ser Pro Ala Lys Leu Leu Leu Gln Met Asp Ser
            1140                1145                1150
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tct gct aca gct tat ggc tcc aca gtt tcc aag agg gtg gca tgg cat     3504
Ser Ala Thr Ala Tyr Gly Ser Thr Val Ser Lys Arg Val Ala Trp His
        1155                1160                1165

tat gat gaa gag aag att gaa ttt gaa tgg aac aca ggc acc aat gta     3552
Tyr Asp Glu Glu Lys Ile Glu Phe Glu Trp Asn Thr Gly Thr Asn Val
    1170                1175                1180

gat acc aaa aaa atg act tcc aat ttc cct gtg gat ctc tcc gat tat     3600
Asp Thr Lys Lys Met Thr Ser Asn Phe Pro Val Asp Leu Ser Asp Tyr
1185                1190                1195                1200

cct aag agc ttg cat atg tat gct aat aga ctc ctg gat cac aga gtc     3648
Pro Lys Ser Leu His Met Tyr Ala Asn Arg Leu Leu Asp His Arg Val
                1205                1210                1215

cct caa aca gac atg act ttc cgg cac gtg ggt tcc aaa tta ata gtt     3696
Pro Gln Thr Asp Met Thr Phe Arg His Val Gly Ser Lys Leu Ile Val
            1220                1225                1230

gca atg agc tca tgg ctt cag aag gca tct ggg agt ctt cct tat acc     3744
Ala Met Ser Ser Trp Leu Gln Lys Ala Ser Gly Ser Leu Pro Tyr Thr
        1235                1240                1245

cag act ttg caa gac cac ctc aat agc ctg aag gag ttc aac ctc cag     3792
Gln Thr Leu Gln Asp His Leu Asn Ser Leu Lys Glu Phe Asn Leu Gln
    1250                1255                1260

aac atg gga ttg cca gac tcc cac atc cca gaa aac ctc ttc tta aaa     3840
Asn Met Gly Leu Pro Asp Ser His Ile Pro Glu Asn Leu Phe Leu Lys
1265                1270                1275                1280

agc gat ggc cgc gtc aaa tat acc ttg aac aag aac agt ttg aaa att     3888
Ser Asp Gly Arg Val Lys Tyr Thr Leu Asn Lys Asn Ser Leu Lys Ile
                1285                1290                1295

gag att cct ttg cct ttt ggt ggc aaa tcc tcc aga gat cta aag atg     3936
Glu Ile Pro Leu Pro Phe Gly Gly Lys Ser Ser Arg Asp Leu Lys Met
            1300                1305                1310

tta gag act gtt agg aca cca gcc ctc cac ttc aag tct gtg gga ttc     3984
Leu Glu Thr Val Arg Thr Pro Ala Leu His Phe Lys Ser Val Gly Phe
        1315                1320                1325

cat ctg cca tct cga gag ttc caa gtc cct act ttt acc att ccc aag     4032
His Leu Pro Ser Arg Glu Phe Gln Val Pro Thr Phe Thr Ile Pro Lys
    1330                1335                1340

ttg tat caa ctg caa gtg cct ctc ctg ggt gtt cta gac ctc tcc acg     4080
Leu Tyr Gln Leu Gln Val Pro Leu Leu Gly Val Leu Asp Leu Ser Thr
1345                1350                1355                1360

aat gtc tac agc aac ttg tac aac tgg tcc gcc tcc tac agt ggt ggc     4128
Asn Val Tyr Ser Asn Leu Tyr Asn Trp Ser Ala Ser Tyr Ser Gly Gly
                1365                1370                1375

aac acc agc aca gac cat ttc agc ctt cgg gct cgt tac cac atg aag     4176
Asn Thr Ser Thr Asp His Phe Ser Leu Arg Ala Arg Tyr His Met Lys
            1380                1385                1390
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gct gac tct gtg gtt gac ctg ctt tcc tac aat gtg caa gga tct gga 4224
Ala Asp Ser Val Val Asp Leu Leu Ser Tyr Asn Val Gln Gly Ser Gly
1395 1400 1405

gaa aca aca tat gac cac aag aat acg ttc aca cta tca tgt gat ggg 4272
Glu Thr Thr Tyr Asp His Lys Asn Thr Phe Thr Leu Ser Cys Asp Gly
1410 1415 1420

tct cta cgc cac aaa ttt cta gat tcg aat atc aaa ttc agt cat gta 4320
Ser Leu Arg His Lys Phe Leu Asp Ser Asn Ile Lys Phe Ser His Val
1425 1430 1435 1440

gaa aaa ctt gga aac aac cca gtc tca aaa ggt tta cta ata ttc gat 4368
Glu Lys Leu Gly Asn Asn Pro Val Ser Lys Gly Leu Leu Ile Phe Asp
1445 1450 1455

gca tct agt tcc tgg gga cca cag atg tct gct tca gtt cat ttg gac 4416
Ala Ser Ser Ser Trp Gly Pro Gln Met Ser Ala Ser Val His Leu Asp
1460 1465 1470

tcc aaa aag aaa cag cat ttg ttt gtc aaa gaa gtc aag att gat ggg 4464
Ser Lys Lys Lys Gln His Leu Phe Val Lys Glu Val Lys Ile Asp Gly
1475 1480 1485

cag ttc aga gtc tct tcg ttc tat gct aaa ggc aca tat ggc ctg tct 4512
Gln Phe Arg Val Ser Ser Phe Tyr Ala Lys Gly Thr Tyr Gly Leu Ser
1490 1495 1500

tgt cag agg gat cct aac act ggc cgg ctc aat gga gag tcc aac ctg 4560
Cys Gln Arg Asp Pro Asn Thr Gly Arg Leu Asn Gly Glu Ser Asn Leu
1505 1510 1515 1520

agg ttt aac tcc tcc tac ctc caa ggc acc aac cag ata aca gga aga 4608
Arg Phe Asn Ser Ser Tyr Leu Gln Gly Thr Asn Gln Ile Thr Gly Arg
1525 1530 1535

tat gaa gat gga acc ctc tcc ctc acc tcc acc tct gat ctg caa agt 4656
Tyr Glu Asp Gly Thr Leu Ser Leu Thr Ser Thr Ser Asp Leu Gln Ser
1540 1545 1550

ggc atc att aaa aat act gct tcc cta aag tat gag aac tac gag ctg 4704
Gly Ile Ile Lys Asn Thr Ala Ser Leu Lys Tyr Glu Asn Tyr Glu Leu
1555 1560 1565

act tta aaa tct gac acc aat ggg aag tat aag aac ttt gcc act tct 4752
Thr Leu Lys Ser Asp Thr Asn Gly Lys Tyr Lys Asn Phe Ala Thr Ser
1570 1575 1580

aac aag atg gat atg acc ttc tct aag caa aat gca ctg ctg cgt tct 4800
Asn Lys Met Asp Met Thr Phe Ser Lys Gln Asn Ala Leu Leu Arg Ser
1585 1590 1595 1600

gaa tat cag gct gat tac gag tca ttg agg ttc ttc agc ctg ctt tct 4848
Glu Tyr Gln Ala Asp Tyr Glu Ser Leu Arg Phe Phe Ser Leu Leu Ser
1605 1610 1615

gga tca cta aat tcc cat ggt ctt gag tta aat gct gac atc tta ggc 4896
Gly Ser Leu Asn Ser His Gly Leu Glu Leu Asn Ala Asp Ile Leu Gly
1620 1625 1630

act gac aaa att aat agt ggt gct cac aag gcg aca cta agg att ggc 4944

Thr Asp Lys Ile Asn Ser Gly Ala His Lys Ala Thr Leu Arg Ile Gly
1635 1640 1645

caa gat gga ata tct acc agt gca acg acc aac ttg aag tgt agt ctc 4992
Gln Asp Gly Ile Ser Thr Ser Ala Thr Thr Asn Leu Lys Cys Ser Leu
1650 1655 1660

ctg gtg ctg gag aat gag ctg aat gca gag ctt ggc ctc tct ggg gca 5040
Leu Val Leu Glu Asn Glu Leu Asn Ala Glu Leu Gly Leu Ser Gly Ala
1665 1670 1675 1680

tct atg aaa tta aca aca aat ggc cgc ttc agg gaa cac aat gca aaa 5088
Ser Met Lys Leu Thr Thr Asn Gly Arg Phe Arg Glu His Asn Ala Lys
1685 1690 1695

ttc agt ctg gat ggg aaa gcc gcc ctc aca gag cta tca ctg gga agt 5136
Phe Ser Leu Asp Gly Lys Ala Ala Leu Thr Glu Leu Ser Leu Gly Ser
1700 1705 1710

gct tat cag gcc atg att ctg ggt gtc gac agc aaa aac att ttc aac 5184
Ala Tyr Gln Ala Met Ile Leu Gly Val Asp Ser Lys Asn Ile Phe Asn
1715 1720 1725

ttc aag gtc agt caa gaa gga ctt aag ctc tca aat gac atg atg ggc 5232
Phe Lys Val Ser Gln Glu Gly Leu Lys Leu Ser Asn Asp Met Met Gly
1730 1735 1740

tca tat gct gaa atg aaa ttt gac cac aca aac agt ctg aac att gca 5280
Ser Tyr Ala Glu Met Lys Phe Asp His Thr Asn Ser Leu Asn Ile Ala
1745 1750 1755 1760

ggc tta tca ctg gac ttc tct tca aaa ctt gac aac att tac agc tct 5328
Gly Leu Ser Leu Asp Phe Ser Ser Lys Leu Asp Asn Ile Tyr Ser Ser
1765 1770 1775

gac aag ttt tat aag caa act gtt aat tta cag cta cag ccc tat tct 5376
Asp Lys Phe Tyr Lys Gln Thr Val Asn Leu Gln Leu Gln Pro Tyr Ser
1780 1785 1790

ctg gta act act tta aac agt gac ctg aaa tac aat gct ctg gat ctc 5424
Leu Val Thr Thr Leu Asn Ser Asp Leu Lys Tyr Asn Ala Leu Asp Leu
1795 1800 1805

acc aac aat ggg aaa cta cgg cta gaa ccc ctg aag ctg cat gtg gct 5472
Thr Asn Asn Gly Lys Leu Arg Leu Glu Pro Leu Lys Leu His Val Ala
1810 1815 1820

ggt aac cta aaa gga gcc tac caa aat aat gaa ata aaa cac atc tat 5520
Gly Asn Leu Lys Gly Ala Tyr Gln Asn Asn Glu Ile Lys His Ile Tyr
1825 1830 1835 1840

gcc atc tct tct gct gcc tta tca gca agc tat aaa gca gac act gtt 5568
Ala Ile Ser Ser Ala Ala Leu Ser Ala Ser Tyr Lys Ala Asp Thr Val
1845 1850 1855

gct aag gtt cag ggt gtg gag ttt agc cat ggg ctc aac aca gac atc 5616
Ala Lys Val Gln Gly Val Glu Phe Ser His Gly Leu Asn Thr Asp Ile
1860 1865 1870

gct ggg ctg gct tca gcc att gac atg agc aca aac tat aat tca gac 5664
Ala Gly Leu Ala Ser Ala Ile Asp Met Ser Thr Asn Tyr Asn Ser Asp
1875 1880 1885

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tca ctg cat ttc agc aat gtc ttc cgt tct gta atg gcc ccg ttt acc    5712
Ser Leu His Phe Ser Asn Val Phe Arg Ser Val Met Ala Pro Phe Thr
    1890                1895                1900

atg acc atc gat gca cat aca aat ggc aat ggg aaa ctc gct ctc tgg    5760
Met Thr Ile Asp Ala His Thr Asn Gly Asn Gly Lys Leu Ala Leu Trp
1905                1910                1915                1920

gga gaa cat act ggg cag ctg tat agc aaa ttc ctg ttg aaa gca gaa    5808
Gly Glu His Thr Gly Gln Leu Tyr Ser Lys Phe Leu Leu Lys Ala Glu
                1925                1930                1935

cct ctg gca ttt act ttc tct cat gat tac aaa ggc tcc aca agt cat    5856
Pro Leu Ala Phe Thr Phe Ser His Asp Tyr Lys Gly Ser Thr Ser His
            1940                1945                1950

cat ctc gtg tct agg aaa agc atc agt gca gct ctt gaa cac aaa gtc    5904
His Leu Val Ser Arg Lys Ser Ile Ser Ala Ala Leu Glu His Lys Val
        1955                1960                1965

agt gcc ctg ctt act cca gct gag cag aca ggc acc tgg aaa ctc aag    5952
Ser Ala Leu Leu Thr Pro Ala Glu Gln Thr Gly Thr Trp Lys Leu Lys
    1970                1975                1980

acc caa ttt aac aac aat gaa tac agc cag gac ttg gat gct tac aac    6000
Thr Gln Phe Asn Asn Asn Glu Tyr Ser Gln Asp Leu Asp Ala Tyr Asn
1985                1990                1995                2000

act aaa gat aaa att ggc gtg gag ctt act gga cga act ctg gct gac    6048
Thr Lys Asp Lys Ile Gly Val Glu Leu Thr Gly Arg Thr Leu Ala Asp
                2005                2010                2015

cta act cta cta gac tcc cca att aaa gtg cca ctt tta ctc agt gag    6096
Leu Thr Leu Leu Asp Ser Pro Ile Lys Val Pro Leu Leu Leu Ser Glu
            2020                2025                2030

ccc atc aat atc aat gat gct tta gag atg aga gat gcc gtt gag aag    6144
Pro Ile Asn Ile Asn Asp Ala Leu Glu Met Arg Asp Ala Val Glu Lys
        2035                2040                2045

ccc caa gaa ttt aca att gtt gct ttt gta aag tat gat aaa aac caa    6192
Pro Gln Glu Phe Thr Ile Val Ala Phe Val Lys Tyr Asp Lys Asn Gln
    2050                2055                2060

gat gtt cac tcc att aac ctc cca ttt ttt gag acc ttg caa gaa tat    6240
Asp Val His Ser Ile Asn Leu Pro Phe Phe Glu Thr Leu Gln Glu Tyr
2065                2070                2075                2080

ttt gag agg aat cga caa acc att ata gtt gta ctg gaa aac gta cag    6288
Phe Glu Arg Asn Arg Gln Thr Ile Ile Val Val Leu Glu Asn Val Gln
                2085                2090                2095

aga aac ctg aag cac atc aat att gat caa ttt gta aga aaa tac aga    6336
Arg Asn Leu Lys His Ile Asn Ile Asp Gln Phe Val Arg Lys Tyr Arg
            2100                2105                2110

gca gcc ctg gga aaa ctc cca cag caa gct aat gat tat ctg aat tca    6384
Ala Ala Leu Gly Lys Leu Pro Gln Gln Ala Asn Asp Tyr Leu Asn Ser
        2115                2120                2125

ttc aat tgg gag aga caa gtt tca cat gcc aag gag aaa ctg act gct    6432
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Phe Asn Trp Glu Arg Gln Val Ser His Ala Lys Glu Lys Leu Thr Ala
    2130                2135                2140

ctc aca aaa aag tat aga att aca gaa aat gat ata caa att gca tta     6480
Leu Thr Lys Lys Tyr Arg Ile Thr Glu Asn Asp Ile Gln Ile Ala Leu
2145                2150                2155                2160

gat gat gcc aaa atc aac ttt aat gaa aaa cta tct caa ctg cag aca     6528
Asp Asp Ala Lys Ile Asn Phe Asn Glu Lys Leu Ser Gln Leu Gln Thr
                2165                2170                2175

tat atg ata caa ttt gat cag tat att aaa gat agt tat gat tta cat     6576
Tyr Met Ile Gln Phe Asp Gln Tyr Ile Lys Asp Ser Tyr Asp Leu His
            2180                2185                2190

gat ttg aaa ata gct att gct aat att att gat gaa atc att gaa aaa     6624
Asp Leu Lys Ile Ala Ile Ala Asn Ile Ile Asp Glu Ile Ile Glu Lys
        2195                2200                2205

tta aaa agt ctt gat gag cac tat cat acc cgt gta aat tta gta aaa     6672
Leu Lys Ser Leu Asp Glu His Tyr His Thr Arg Val Asn Leu Val Lys
    2210                2215                2220

aca atc cat gat cta cat ttg ttt att gaa aat att gat ttt aac aaa     6720
Thr Ile His Asp Leu His Leu Phe Ile Glu Asn Ile Asp Phe Asn Lys
2225                2230                2235                2240

agt gga agt agt act gca tcc tgg att caa aat gtg gat act aag tac     6768
Ser Gly Ser Ser Thr Ala Ser Trp Ile Gln Asn Val Asp Thr Lys Tyr
                2245                2250                2255

caa atc aga atc cag ata caa gaa aaa ctg cag cag ctt aag aga cac     6816
Gln Ile Arg Ile Gln Ile Gln Glu Lys Leu Gln Gln Leu Lys Arg His
            2260                2265                2270

ata cag aat ata gac atc cag cac cta gct gga aag tta aaa caa cac     6864
Ile Gln Asn Ile Asp Ile Gln His Leu Ala Gly Lys Leu Lys Gln His
        2275                2280                2285

att gag gct att gat gtt aga gtg ctt tta gat caa ttg gga act aca     6912
Ile Glu Ala Ile Asp Val Arg Val Leu Leu Asp Gln Leu Gly Thr Thr
    2290                2295                2300

att tca ttt gaa aga ata aat gat gtt ctt gag cat gtc aaa cac ttt     6960
Ile Ser Phe Glu Arg Ile Asn Asp Val Leu Glu His Val Lys His Phe
2305                2310                2315                2320

gtt ata aat ctt att ggg gat ttt gaa gta gct gag aaa atc aat gcc     7008
Val Ile Asn Leu Ile Gly Asp Phe Glu Val Ala Glu Lys Ile Asn Ala
                2325                2330                2335

ttc aga gcc aaa gtc cat gag tta atc gag agg tat gaa gta gac caa     7056
Phe Arg Ala Lys Val His Glu Leu Ile Glu Arg Tyr Glu Val Asp Gln
            2340                2345                2350

caa atc cag gtt tta atg gat aaa tta gta gag ttg gcc cac caa tac     7104
Gln Ile Gln Val Leu Met Asp Lys Leu Val Glu Leu Ala His Gln Tyr
        2355                2360                2365

aag ttg aag gag act att cag aag cta agc aat gtc cta caa caa gtt     7152
Lys Leu Lys Glu Thr Ile Gln Lys Leu Ser Asn Val Leu Gln Gln Val
    2370                2375                2380
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aag ata aaa gat tac ttt gag aaa ttg gtt gga ttt att gat gat gct 7200
Lys Ile Lys Asp Tyr Phe Glu Lys Leu Val Gly Phe Ile Asp Asp Ala
2385 2390 2395 2400

gtc aag aag ctt aat gaa tta tct ttt aaa aca ttc att gaa gat gtt 7248
Val Lys Lys Leu Asn Glu Leu Ser Phe Lys Thr Phe Ile Glu Asp Val
2405 2410 2415

aac aaa ttc ctt gac atg ttg ata aag aaa tta aag tca ttt gat tac 7296
Asn Lys Phe Leu Asp Met Leu Ile Lys Lys Leu Lys Ser Phe Asp Tyr
2420 2425 2430

cac cag ttt gta gat gaa acc aat gac aaa atc cgt gag gtg act cag 7344
His Gln Phe Val Asp Glu Thr Asn Asp Lys Ile Arg Glu Val Thr Gln
2435 2440 2445

aga ctc aat ggt gaa att cag gct ctg gaa cta cca caa aaa gct gaa 7392
Arg Leu Asn Gly Glu Ile Gln Ala Leu Glu Leu Pro Gln Lys Ala Glu
2450 2455 2460

gca tta aaa ctg ttt tta gag gaa acc aag gcc aca gtt gca gtg tat 7440
Ala Leu Lys Leu Phe Leu Glu Glu Thr Lys Ala Thr Val Ala Val Tyr
2465 2470 2475 2480

ctg gaa agc cta cag gac acc aaa ata acc tta atc atc aat tgg tta 7488
Leu Glu Ser Leu Gln Asp Thr Lys Ile Thr Leu Ile Ile Asn Trp Leu
2485 2490 2495

cag gag gct tta agt tca gca tct ttg gct cac atg aag gcc aaa ttc 7536
Gln Glu Ala Leu Ser Ser Ala Ser Leu Ala His Met Lys Ala Lys Phe
2500 2505 2510

cga gag act cta gaa gat aca cga gac cga atg tat caa atg gac att 7584
Arg Glu Thr Leu Glu Asp Thr Arg Asp Arg Met Tyr Gln Met Asp Ile
2515 2520 2525

cag cag gaa ctt caa cga tac ctg tct ctg gta ggc cag gtt tat agc 7632
Gln Gln Glu Leu Gln Arg Tyr Leu Ser Leu Val Gly Gln Val Tyr Ser
2530 2535 2540

aca ctt gtc acc tac att tct gat tgg tgg act ctt gct gct aag aac 7680
Thr Leu Val Thr Tyr Ile Ser Asp Trp Trp Thr Leu Ala Ala Lys Asn
2545 2550 2555 2560

ctt act gac ttt gca gag caa tat tct atc caa gat tgg gct aaa cgt 7728
Leu Thr Asp Phe Ala Glu Gln Tyr Ser Ile Gln Asp Trp Ala Lys Arg
2565 2570 2575

atg aaa gca ttg gta gag caa ggg ttc act gtt cct gaa atc aag acc 7776
Met Lys Ala Leu Val Glu Gln Gly Phe Thr Val Pro Glu Ile Lys Thr
2580 2585 2590

atc ctt ggg acc atg cct gcc ttt gaa gtc agt ctt cag gct ctt cag 7824
Ile Leu Gly Thr Met Pro Ala Phe Glu Val Ser Leu Gln Ala Leu Gln
2595 2600 2605

aaa gct acc ttc cag aca cct gat ttt ata gtc ccc cta aca gat ttg 7872
Lys Ala Thr Phe Gln Thr Pro Asp Phe Ile Val Pro Leu Thr Asp Leu
2610 2615 2620

agg att cca tca gtt cag ata aac ttc aaa gac tta aaa aat ata aaa 7920

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Arg Ile Pro Ser Val Gln Ile Asn Phe Lys Asp Leu Lys Asn Ile Lys
2625                2630                2635                2640

atc cca tcc agg ttt tcc aca cca gaa ttt acc atc ctt aac acc ttc      7968
Ile Pro Ser Arg Phe Ser Thr Pro Glu Phe Thr Ile Leu Asn Thr Phe
                2645                2650                2655

cac att cct tcc ttt aca att gac ttt gta gaa atg aaa gta aag atc      8016
His Ile Pro Ser Phe Thr Ile Asp Phe Val Glu Met Lys Val Lys Ile
            2660                2665                2670

atc aga acc att gac cag atg ctg aac agt gag ctg cag tgg ccc gtt      8064
Ile Arg Thr Ile Asp Gln Met Leu Asn Ser Glu Leu Gln Trp Pro Val
        2675                2680                2685

cca gat ata tat ctc agg gat ctg aag gtg gag gac att cct cta gcg      8112
Pro Asp Ile Tyr Leu Arg Asp Leu Lys Val Glu Asp Ile Pro Leu Ala
    2690                2695                2700

aga atc acc ctg cca gac ttc cgt tta cca gaa atc gca att cca gaa      8160
Arg Ile Thr Leu Pro Asp Phe Arg Leu Pro Glu Ile Ala Ile Pro Glu
2705                2710                2715                2720

ttc ata atc cca act ctc aac ctt aat gat ttt caa gtt cct gac ctt      8208
Phe Ile Ile Pro Thr Leu Asn Leu Asn Asp Phe Gln Val Pro Asp Leu
                2725                2730                2735

cac ata cca gaa ttc cag ctt ccc cac atc tca cac aca att gaa gta      8256
His Ile Pro Glu Phe Gln Leu Pro His Ile Ser His Thr Ile Glu Val
            2740                2745                2750

cct act ttt ggc aag cta tac agt att ctg aaa atc caa tct cct ctt      8304
Pro Thr Phe Gly Lys Leu Tyr Ser Ile Leu Lys Ile Gln Ser Pro Leu
        2755                2760                2765

ttc aca tta gat gca aat gct gac ata ggg aat gga acc acc tca gca      8352
Phe Thr Leu Asp Ala Asn Ala Asp Ile Gly Asn Gly Thr Thr Ser Ala
    2770                2775                2780

aac gaa gca ggt atc gca gct tcc atc act gcc aaa gga gag tcc aaa      8400
Asn Glu Ala Gly Ile Ala Ala Ser Ile Thr Ala Lys Gly Glu Ser Lys
2785                2790                2795                2800

tta gaa gtt ctc aat ttt gat ttt caa gca aat gca caa ctc tca aac      8448
Leu Glu Val Leu Asn Phe Asp Phe Gln Ala Asn Ala Gln Leu Ser Asn
                2805                2810                2815

cct aag att aat ccg ctg gct ctg aag gag tca gtg aag ttc tcc agc      8496
Pro Lys Ile Asn Pro Leu Ala Leu Lys Glu Ser Val Lys Phe Ser Ser
            2820                2825                2830

aag tac ctg aga acg gag cat ggg agt gaa atg ctg ttt ttt gga aat      8544
Lys Tyr Leu Arg Thr Glu His Gly Ser Glu Met Leu Phe Phe Gly Asn
        2835                2840                2845

gct att gag gga aaa tca aac aca gtg gca agt tta cac aca gaa aaa      8592
Ala Ile Glu Gly Lys Ser Asn Thr Val Ala Ser Leu His Thr Glu Lys
    2850                2855                2860

aat aca ctg gag ctt agt aat gga gtg att gtc aag ata aac aat cag      8640
Asn Thr Leu Glu Leu Ser Asn Gly Val Ile Val Lys Ile Asn Asn Gln
2865                2870                2875                2880
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ctt acc ctg gat agc aac act aaa tac ttc cac aaa ttg aac atc ccc 8688
Leu Thr Leu Asp Ser Asn Thr Lys Tyr Phe His Lys Leu Asn Ile Pro
2885 2890 2895

aaa ctg gac ttc tct agt cag gct gac ctg cgc aac gag atc aag aca 8736
Lys Leu Asp Phe Ser Ser Gln Ala Asp Leu Arg Asn Glu Ile Lys Thr
2900 2905 2910

ctg ttg aaa gct ggc cac ata gca tgg act tct tct gga aaa ggg tca 8784
Leu Leu Lys Ala Gly His Ile Ala Trp Thr Ser Ser Gly Lys Gly Ser
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tgg aaa tgg gcc tcg ccc aga ttc tca gat gag gga aca cat gaa tca 8832
Trp Lys Trp Ala Ser Pro Arg Phe Ser Asp Glu Gly Thr His Glu Ser
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caa att agt ttc acc ata gaa gga ccc ctc act tcc ttt gga ctg tcc 8880
Gln Ile Ser Phe Thr Ile Glu Gly Pro Leu Thr Ser Phe Gly Leu Ser
2945 2950 2955 2960

aat aag atc aat agc aaa cac cta aga gta aac caa aac ttg gtt tat 8928
Asn Lys Ile Asn Ser Lys His Leu Arg Val Asn Gln Asn Leu Val Tyr
2965 2970 2975

gaa tct ggc tcc ctc aac ttt tct aaa ctt gaa att caa tca caa gtc 8976
Glu Ser Gly Ser Leu Asn Phe Ser Lys Leu Glu Ile Gln Ser Gln Val
2980 2985 2990

gat tcc cag cat gtg ggc cac agt gtt cta act gct aaa ggc atg gca 9024
Asp Ser Gln His Val Gly His Ser Val Leu Thr Ala Lys Gly Met Ala
2995 3000 3005

ctg ttt gga gaa ggg aag gca gag ttt act ggg agg cat gat gct cat 9072
Leu Phe Gly Glu Gly Lys Ala Glu Phe Thr Gly Arg His Asp Ala His
3010 3015 3020

tta aat gga aag gtt att gga act ttg aaa aat tct ctt ttc ttt tca 9120
Leu Asn Gly Lys Val Ile Gly Thr Leu Lys Asn Ser Leu Phe Phe Ser
3025 3030 3035 3040

gcc cag cca ttt gag atc acg gca tcc aca aac aat gaa ggg aat ttg 9168
Ala Gln Pro Phe Glu Ile Thr Ala Ser Thr Asn Asn Glu Gly Asn Leu
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Lys Val Arg Phe Pro Leu Arg Leu Thr Gly Lys Ile Asp Phe Leu Asn
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aac tat gca ctg ttt ctg agt ccc agt gcc cag caa gca agt tgg caa 9264
Asn Tyr Ala Leu Phe Leu Ser Pro Ser Ala Gln Gln Ala Ser Trp Gln
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gta agt gct agg ttc aat cag tat aag tac aac caa aat ttc tct gct 9312
Val Ser Ala Arg Phe Asn Gln Tyr Lys Tyr Asn Gln Asn Phe Ser Ala
3090 3095 3100

gga aac aac gag aac att atg gag gcc cat gta gga ata aat gga gaa 9360
Gly Asn Asn Glu Asn Ile Met Glu Ala His Val Gly Ile Asn Gly Glu
3105 3110 3115 3120

gca aat ctg gat ttc tta aac att cct tta aca att cct gaa atg cgt 9408

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Ala Asn Leu Asp Phe Leu Asn Ile Pro Leu Thr Ile Pro Glu Met Arg
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cta cct tac aca ata atc aca act cct cca ctg aaa gat ttc tct cta      9456
Leu Pro Tyr Thr Ile Ile Thr Thr Pro Pro Leu Lys Asp Phe Ser Leu
            3140                3145                3150

tgg gaa aaa aca ggc ttg aag gaa ttc ttg aaa acg aca aag caa tca      9504
Trp Glu Lys Thr Gly Leu Lys Glu Phe Leu Lys Thr Thr Lys Gln Ser
        3155                3160                3165

ttt gat tta agt gta aaa gct cag tat aag aaa aac aaa cac agg cat      9552
Phe Asp Leu Ser Val Lys Ala Gln Tyr Lys Lys Asn Lys His Arg His
    3170                3175                3180

tcc atc aca aat cct ttg gct gtg ctt tgt gag ttt atc agt cag agc      9600
Ser Ile Thr Asn Pro Leu Ala Val Leu Cys Glu Phe Ile Ser Gln Ser
3185                3190                3195                3200

atc aaa tcc ttt gac agg cat ttt gaa aaa aac aga aac aat gca tta      9648
Ile Lys Ser Phe Asp Arg His Phe Glu Lys Asn Arg Asn Asn Ala Leu
                3205                3210                3215

gat ttt gtc acc aaa tcc tat aat gaa aca aaa att aag ttt gat aag      9696
Asp Phe Val Thr Lys Ser Tyr Asn Glu Thr Lys Ile Lys Phe Asp Lys
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tac aaa gct gaa aaa tct cac gac gag ctc ccc agg acc ttt caa att      9744
Tyr Lys Ala Glu Lys Ser His Asp Glu Leu Pro Arg Thr Phe Gln Ile
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cct gga tac act gtt cca gtt gtc aat gtt gaa gtg tct cca ttc acc      9792
Pro Gly Tyr Thr Val Pro Val Val Asn Val Glu Val Ser Pro Phe Thr
    3250                3255                3260

ata gag atg tcg gca ttc ggc tat gtg ttc cca aaa gca gtc agc atg      9840
Ile Glu Met Ser Ala Phe Gly Tyr Val Phe Pro Lys Ala Val Ser Met
3265                3270                3275                3280

cct agt ttc tcc atc ata ggt tct gac gtc cgt gtg cct tca tac aca      9888
Pro Ser Phe Ser Ile Ile Gly Ser Asp Val Arg Val Pro Ser Tyr Thr
                3285                3290                3295

tta atc ctg cca tca tta gag ctg cca gtc ctt cat gtc cct aga aat      9936
Leu Ile Leu Pro Ser Leu Glu Leu Pro Val Leu His Val Pro Arg Asn
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Leu Lys Leu Ser Leu Pro Asp Phe Lys Glu Leu Cys Thr Ile Ser His
        3315                3320                3325

att ttt att cct gcc atg ggc aat att acc tat gat ttc tcc ttt aaa      10032
Ile Phe Ile Pro Ala Met Gly Asn Ile Thr Tyr Asp Phe Ser Phe Lys
    3330                3335                3340

tca agt gtc atc aca ctg aat acc aat gct gaa ctt ttt aac cag tca      10080
Ser Ser Val Ile Thr Leu Asn Thr Asn Ala Glu Leu Phe Asn Gln Ser
3345                3350                3355                3360

gat att gtt gct cat ctc ctt tct tca tct tca tct gtc att gat gca      10128
Asp Ile Val Ala His Leu Leu Ser Ser Ser Ser Ser Val Ile Asp Ala
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Leu Lys Leu Ala Thr Ala Leu Ser Leu Ser Asn Lys Phe Val Glu Gly
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Ser His Asn Ser Thr Val Ser Leu Thr Thr Lys Asn Met Glu Val Ser
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Val Ala Lys Thr Thr Lys Pro Glu Ile Pro Ile Leu Arg Met Asn Phe
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Lys Gln Glu Leu Asn Gly Asn Thr Lys Ser Lys Pro Thr Val Ser Ser
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Ala Lys Gly Ala Val Asp His Lys Leu Ser Leu Glu Ser Leu Thr Ser
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Tyr Phe Ser Ile Glu Ser Ser Thr Lys Gly Asp Val Lys Gly Ser Val
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Leu Ser Arg Glu Tyr Ser Gly Thr Ile Ala Ser Glu Ala Asn Thr Tyr
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Lys Ile Asp Asp Ile Trp Asn Leu Glu Val Lys Glu Asn Phe Ala Gly
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Ser Lys Ala Thr Leu Glu Leu Ser Pro Trp Gln Met Ser Ala Leu Val
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Gln Val His Ala Ser Gln Pro Ser Ser Phe His Asp Phe Pro Asp Leu
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Trp Lys Asn Glu Val Arg Ile His Ser Gly Ser Phe Gln Ser Gln Val
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Glu Leu Ser Asn Asp Gln Glu Lys Ala His Leu Asp Ile Ala Gly Ser
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Leu Glu Gly His Leu Arg Phe Leu Lys Asn Ile Ile Leu Pro Val Tyr
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Asp Lys Ser Leu Trp Asp Phe Leu Lys Leu Asp Val Thr Thr Ser Ile
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Gly Arg Arg Gln His Leu Arg Val Ser Thr Ala Phe Val Tyr Thr Lys
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Asn Pro Asn Gly Tyr Ser Phe Ser Ile Pro Val Lys Val Leu Ala Asp
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Lys Phe Ile Ile Pro Gly Leu Lys Leu Asn Asp Leu Asn Ser Val Leu
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gtc atg cct acg ttc cat gtc cca ttt aca gat ctt cag gtt cca tcg   11280
Val Met Pro Thr Phe His Val Pro Phe Thr Asp Leu Gln Val Pro Ser
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Cys Lys Leu Asp Phe Arg Glu Ile Gln Ile Tyr Lys Lys Leu Arg Thr
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Ser Ser Phe Ala Leu Thr Leu Pro Thr Leu Pro Glu Val Lys Phe Pro
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gaa gtt gat gtg tta aca aaa tat tct caa cca gaa gac tcc ttg att   11424
Glu Val Asp Val Leu Thr Lys Tyr Ser Gln Pro Glu Asp Ser Leu Ile
        3795                3800                3805

ccc ttt ttt gag ata acc gtg cct gaa tct cag tta act gtg tcc cag   11472
Pro Phe Phe Glu Ile Thr Val Pro Glu Ser Gln Leu Thr Val Ser Gln
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Phe Thr Leu Pro Lys Ser Val Ser Asp Gly Ile Ala Ala Leu Asp Leu
3825                3830                3835                3840

aat gca gta gcc aac aag atc gca gac ttt gag ttg ccc acc atc atc   11568
Asn Ala Val Ala Asn Lys Ile Ala Asp Phe Glu Leu Pro Thr Ile Ile
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Val Pro Glu Gln Thr Ile Glu Ile Pro Ser Ile Lys Phe Ser Val Pro
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Val Asp Ser Pro Val Tyr Asn Ala Thr Trp Ser Ala Ser Leu Lys Asn
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aaa gca gat tat gtt gaa aca gtc ctg gat tcc aca tgc agc tca acc    11760
Lys Ala Asp Tyr Val Glu Thr Val Leu Asp Ser Thr Cys Ser Ser Thr
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Val Gln Phe Leu Glu Tyr Glu Leu Asn Val Leu Gly Thr His Lys Ile
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Glu Asp Gly Thr Leu Ala Ser Lys Thr Lys Gly Thr Leu Ala His Arg
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Asp Phe Ser Ala Glu Tyr Glu Glu Asp Gly Lys Tyr Glu Gly Leu Gln
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Glu Trp Glu Gly Lys Ala His Leu Asn Ile Lys Ser Pro Ala Phe Thr
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Asp Leu His Leu Arg Tyr Gln Lys Asp Lys Lys Gly Ile Ser Thr Ser
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Ala Ala Ser Pro Ala Val Gly Thr Val Gly Met Asp Met Asp Glu Asp
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Asp Asp Phe Ser Lys Trp Asn Phe Tyr Tyr Ser Pro Gln Ser Ser Pro
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Asp Lys Lys Leu Thr Ile Phe Lys Thr Glu Leu Arg Val Arg Glu Ser
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gat gag gaa act cag atc aaa gtt aat tgg gaa gaa gag gca gct tct    12192
Asp Glu Glu Thr Gln Ile Lys Val Asn Trp Glu Glu Glu Ala Ala Ser
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ggc ttg cta acc tct ctg aaa gac aac gtg ccc aag gcc aca ggg gtc    12240
Gly Leu Leu Thr Ser Leu Lys Asp Asn Val Pro Lys Ala Thr Gly Val
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Leu Tyr Asp Tyr Val Asn Lys Tyr His Trp Glu His Thr Gly Leu Thr
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ctg aga gaa gtg tct tca aag ctg aga aga aat ctg cag aac aat gct    12336
Leu Arg Glu Val Ser Ser Lys Leu Arg Arg Asn Leu Gln Asn Asn Ala
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Lys Asp Lys Ala Gln Asn Leu Tyr Gln Glu Leu Leu Thr Gln Glu Gly
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Cys Leu Asn Leu His Lys Phe Asn Glu Phe Ile Gln Asn Glu Leu Gln

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Tyr Glu Leu Glu Glu Lys Ile Val Ser Leu Ile Lys Asn Leu Leu Val
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<210> 795
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<210> 802
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<210> 803
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<213> H. sapiens

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<210> 804
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<210> 805
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<400> 806
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<210> 807
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<220>
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<400> 807
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<210> 808
<211> 432
<212> DNA
<213> O. cuniculus

<400> 808
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tcactgaggt tcttcaccct gctttctggg ttgttgaata cccatggtct tgaattaaat 120

gctgacatct tgggcactga caaaatgaat actgctgctc acaaggcaac tctaagaatt 180

ggccaaaatg gagtatctac cagtgcaaca accagcttga ggtacagtcc cctgatgctg 240

gagaatgagc tgaacgcaga gcttgccctt tctggggcat ctatgaaatt agcaacaaat 300

ggccgcttca aggaacacaa tgcaaaattc agcctagatg ggaaagctac cctcacagag 360

ttatccctgg gaagcgctta ccaggccatg attctgggtg ctgacagcaa gaacattttc 420

aacttcaaga tc 432

<210> 809
<211> 660
<212> DNA
<213> O. cuniculus

<400> 809
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gtttccagtg ccaaggagaa actaactact ttcacaaaaa attataaaat tacagagaat 120

gatatacaaa ctgcattgga taatgccaaa atcaacttaa atgaaaaact gtctcaactt 180

cagacatatg tgatataatt tgatcagtat attaaagata attttgatct acatgatttt 240

aaaatagcta tagctagtat tatagatcaa atcatggaaa aattaaaaat tcttgatgaa 300

cgttatcata tccgtgcaca tttaattaaa tcaatccata atttatattt gtttattgaa 360

gctattgatt ttaacaaaat tggaagtagt actgcatctt ggattcaaaa tgtggatacc 420

aagtatcaag tcagaatctg gatacaagaa atattgcaac agtttaagac acagattcag 480

aatacaaaca tcccatacct ggctgaaaaa ctgaaacaac agattgaggc tattgatgtc 540

agagtgcttt tagatcaatt gagaactaca attccatttc gtataataaa ggacattatt 600

gaacatttca aatactttgt tataaatatt attgaaaatt ttgaagtaat tgacaaaatc 660

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<223> n = a, c, g, or t

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<222> (180)
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cctggataca ttattccaat tttcaatntt gaagtatctc cactcacaat agnagacgtn 180

agcattcagt catgtgatcc caaaatcaat aagcaccccc aatgtcacca tcctggattc 240

aagcttctat gtgccttcat atacattggc tctgccatcc ctagagctgc cagtcttcca 300

tgtccccagg aatctactca aggtctctct tccagatttc aaggaattga aaaccattaa 360

caatattttt attccagcca tgggcaacat tacctatgaa ttttccttca aatcaacgat 420

cattacactg aataccaatg ctggacttta taaccaatca gacattgttg cccatatcct 480

ttcttcctct tcatctgtca ttgatgcact acagtacaaa ttagagggca cgctcaagtt 540

tga 543

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<220>
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aagcaccccc aatgtcacc 19

<210> 812
<211> 20
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<220>
<223> Primer

<400> 812
gggatggcag agccaatgta 20

<210> 813
<211> 29
<212> DNA
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<220>
<223> Probe

<400> 813
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<220>
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<400> 814
tgcttggaga aggtaagatc 20

<210> 815
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<400> 815
gcgttgtctc cgatgttctg 20

<210> 816
<211> 20
<212> DNA
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<220>
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<400> 816
taatcattaa cttgctgtgg 20

<210> 817
<211> 20
<212> DNA
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<220>
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<210> 818
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<220>
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<210> 819
<211> 20
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<220>
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<210> 820
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<400> 820
acctcagtga cttgtaatca 20

<210> 821
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<400> 821
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<210> 822
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<400> 822
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<210> 823
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<400> 824
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<210> 826
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<220>
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<400> 827
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gtagatactc catttggcc 20

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<210> 832
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<400> 832
ggactgtacc tcaagctggt 20

<210> 833
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<210> 838
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<210> 839
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<400> 841
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<210> 842
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<210> 844
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<400> 847
taaagtccag cattggtatt 20

<210> 848
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<400> 849
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<210> 850
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<400> 850
tgacagatga agaggaagaa 20

<210> 851

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<400> 852
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<220>
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<400> 853
acttgagcgt gccctctaat 20

<210> 854
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<220>
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<210> 855
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<213> M. fascicularis

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<222> (7)
<223> n = A,T,C or G

<220>
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<222>(469)..(474)
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<221> misc_feature
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aggacagagc cttggtggac accctgaagt ttgtaactca agcagaaggt gtaaagcaga 180

ctgaggctac catgacattc aaatataatc ggcagagtat gaccttgtcc agtgaagtcc 240

aaattccgga ttttgaggtt gaccttggaa caatcctcag agttaatgat gaatctactg 300

agggcagaaa gtcttacaga ctcaccctgg acattcagaa ccagaaaatt actgaggtca 360

ccctcatggg ccacctaagt tgtgacacaa aggaagaagg aaaaatcaaa ggtgttattt 420

ccgtaccccg tttgcaagca gaagccagaa gtgagatcct cgcccacann nnnnannnn 479

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<210> 858
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<220>
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<400> 858
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<210> 859
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<220>
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caggattgct accatggtga tggacagagt tatcgaggca cgtactccac cactgtcaca 180
ggaaggacct gccaagcttg gtcatctatg acaccacatc aacataatag gaccacagaa 240
aactacccaa atgctggctt gatcatgaac tactgcagga atccagatgc tgtggcagct 300
ccttattgtt atacgaggga tcccggtgtc aggtgggagt actgcaacct gacgcaatgc 360
tcagacgcag aagggactgc cgtcgcgcct ccgactgtta ccccggttcc aagcctagag 420
gctccttccg aacaagcacc gactgagcaa aggcctgggg tgcaggagtg ctaccatggt 480
aatggacaga gttatcgagg cacatactcc accactgtca caggaagaac ctgccaagct 540
tggtcatcta tgacaccaca ctcgcatagt cggaccccag aatactaccc aaatgctggc 600
ttgatcatga actactgcag gaatccagat gctgtggcag ctccttattg ttatacgagg 660
gatcccggtg tcaggtggga gtactgcaac ctgacgcaat gctcagacgc agaagggact 720
gccgtcgcgc ctccgactgt taccccggtt ccaagcctag aggctccttc cgaacaagca 780
ccgactgagc aaaggcctgg ggtgcaggag tgctaccatg gtaatggaca gagttatcga 840
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gtcttccatt ctgtaatggc tccatttacc atgaccattg atacacatac aaatggcaac 300
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gcagaacctc tggcattcac tttctctcat gattacaaag gctccacgag tcatcatctc 420
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/36411

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 48/00; C12Q 1/68; C07H 21/00
US CL : 514/44; 435/6, 325, 375; 536/23.1, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 435/6, 325, 375; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
West, Biosis, Medline, SciSearch, CA, Embase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAW et al. Hunam Apolipoprotein B-100: Cloning, analysis of Liver mRNA and Assignment of the Gene to Chromosome 2. Proc. Natl. Acad. Sci. December 1985, Vol. 82, pages 8340-8344. Throughout, as directed to the use of the cDNA for Human Apolipoprotein B-100.	1-63, 66-72
Y	WO 01/12789 A2 (CHAN et al.) 22 February 2001 (22.02.2001). Throughout.	1-63, 66-72
Y	US 5,801,154 A (BARRACCHINI et al.) 01 September 1998 (01.09.1998). Throughout.	1-63, 66-72

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 August 2004 (09.08.2004)	31 AUG 2004
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 872-9306	Authorized officer J. D. Schultz, Ph.D. F. Roberts for Telephone No. 571-272-1600

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/36411

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 64, 65, 97, 104-127, and 141-144
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.